CHAPTER IV

RESULTS

1. Particle characterization

1.1 Polyester microparticles

1.1.1 Shape and surface morphology

The microparticles produced from DL-PLA, PLGA 85:15, PLGA 75:25 and PLGA 50:50 which were characterized from both unloaded and loaded particles were similar in appearance. The spherical shape of microparticles were observed under optical microscope with camera. Moreover, SEM micrographs showed both spherical shape and smooth surface without pores for all batches of microparticles as depicted in figure 29. Polymer molecular weight and copolymer composition did not affect the appearance of microparticles.

1.1.2 Particle size and size distribution

The particle size of PLGA microparticles characterized by laser particle size analyser is shown in Table 20. The volume mean particle size (D(v,0.5)) of microparticles produced by solvent evaporation method was less than 100 μm. The size distribution of microparticles was rather varied among each preparation. It was shown that the size of microparticles was affected by shearing force used to produce primary emulsion, copolymer composition and polymer concentration and variably affected by the amount of antigen added in preparations. Higher shearing force yielded a smaller particle size than lower shearing force. Different copolymer composition gave particles of different size. Microparticles produced from DL-PLA exhibited larger size than from PLGA 85:15, PLGA 75:25 and PLGA 50:50, respectively. Moreover, particle size increased by increasing the concentration of polymer. Microparticles produced from 5% of polymer concentration exhibited larger size than those produced from

A)

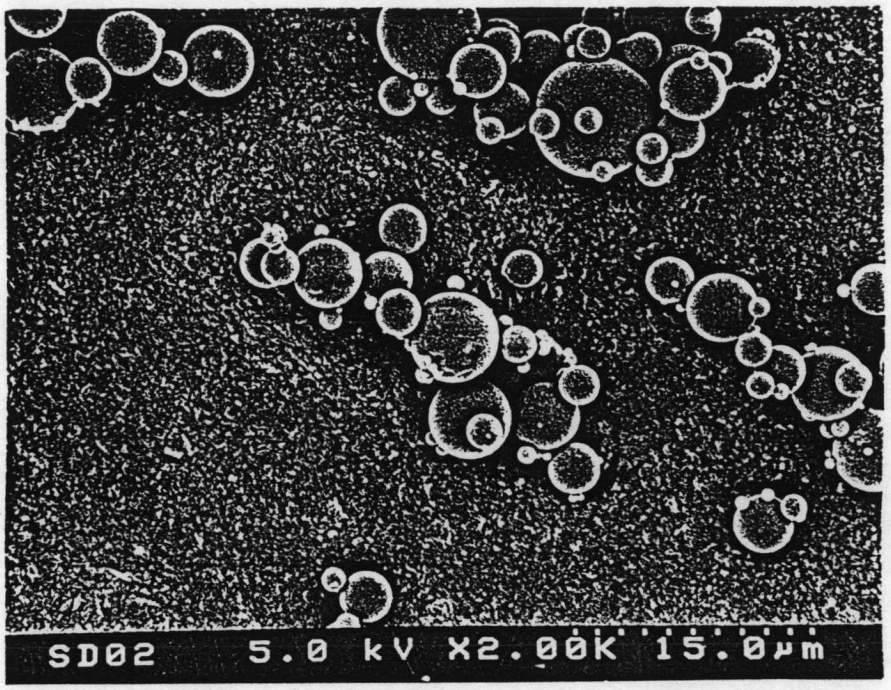


Figure 29 SEM micrographs of unloaded and loaded PLGA microparticles: A) unloaded microparticles, B) loaded microparticles of DL-PLA, C) loaded microparticles of PLGA 85:15, D) loaded microparticles of PLGA 75:25, E) loaded microparticles of PLGA 50:50

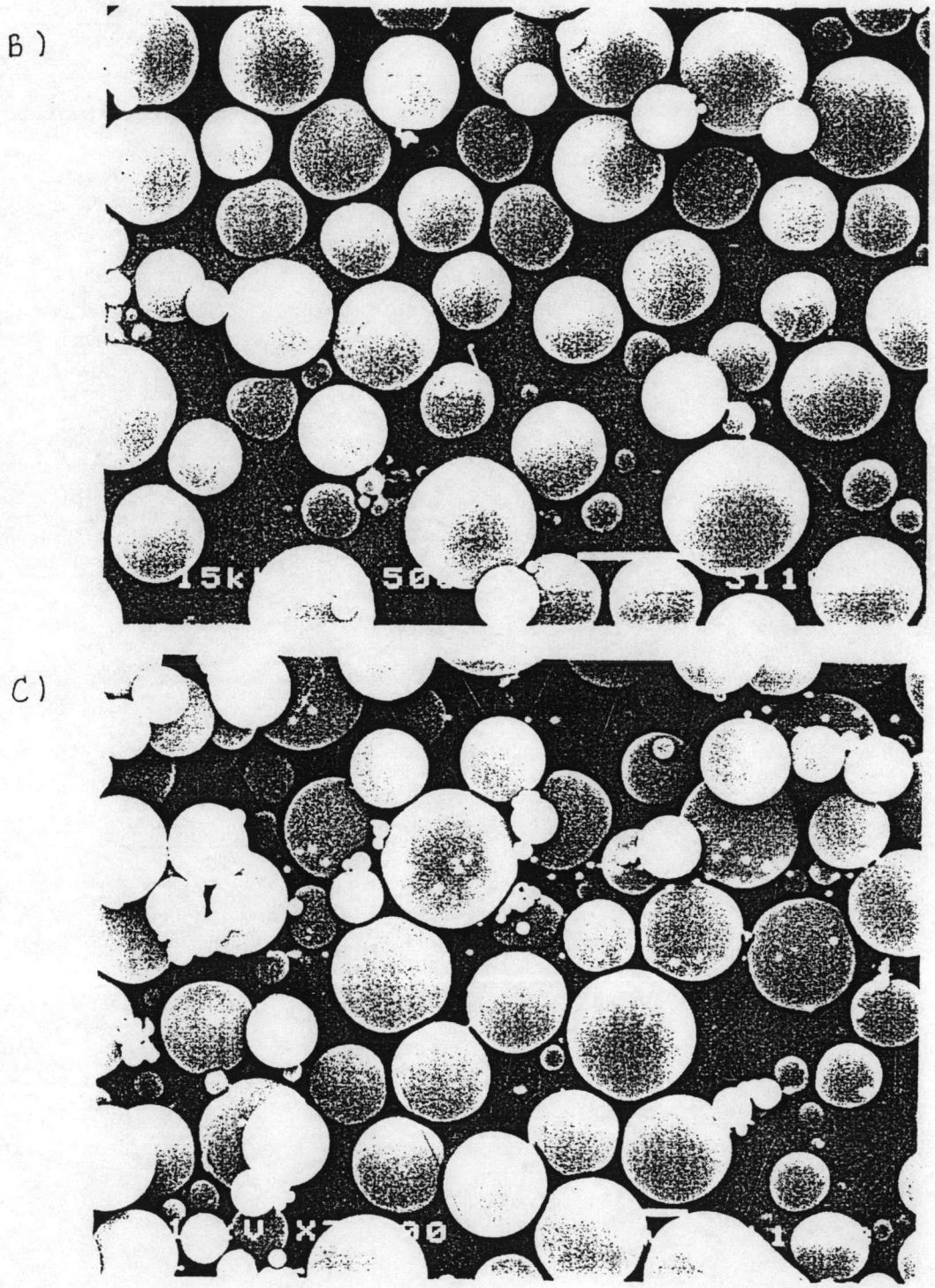


Figure 29 SEM micrographs of unloaded and loaded PLGA microparticles : A) unloaded microparticles , B) loaded microparticles of DL-PLA , C) loaded microparticles of PLGA 85:15 , D) loaded microparticles of PLGA 75:25 , E) loaded microparticles of PLGA 50:50

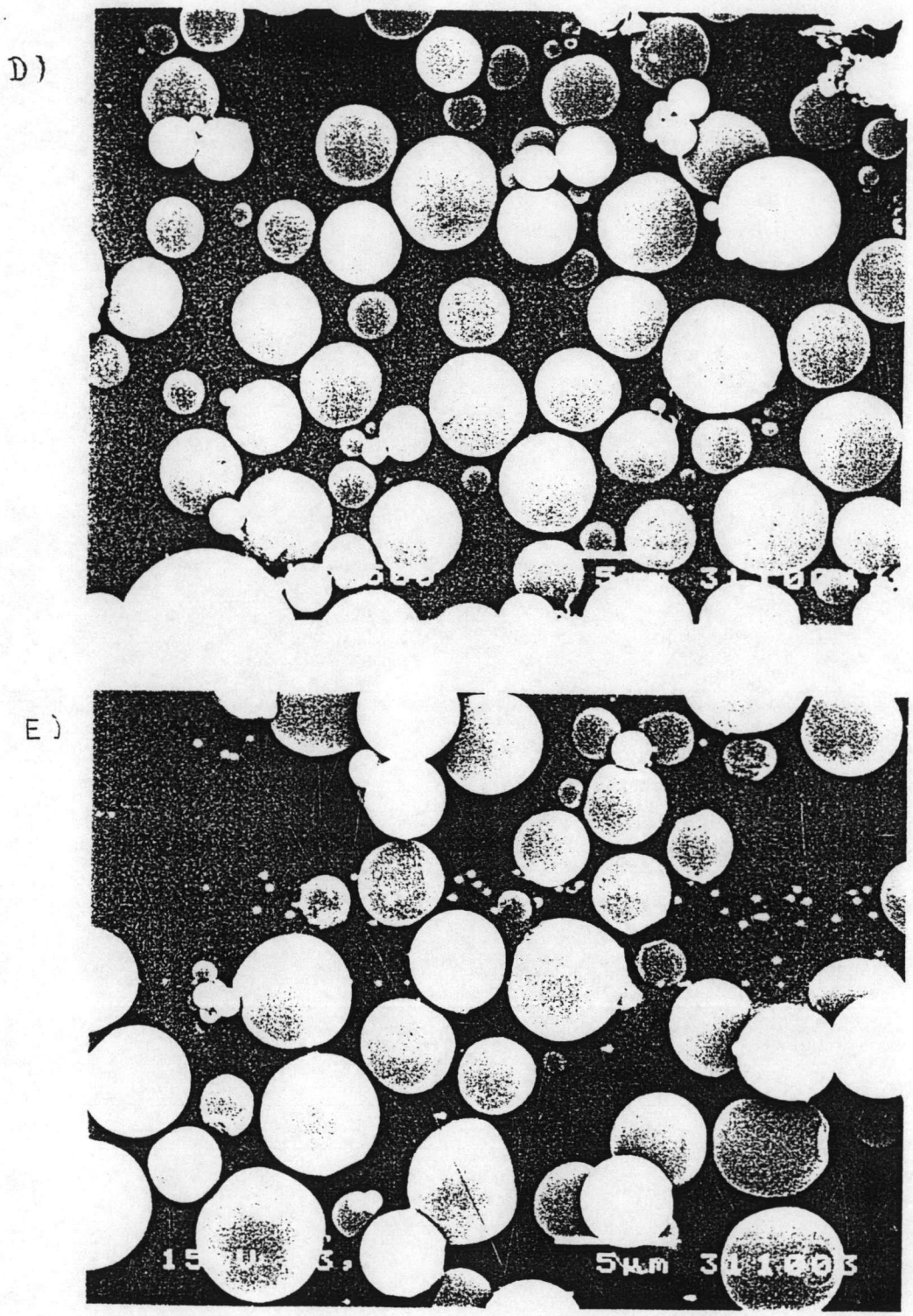


Figure 29 SEM micrographs of unloaded and loaded PLGA microparticles : A) unloaded microparticles , B) loaded microparticles of DL-PLA , C) loaded microparticles of PLGA 85:15 , D) loaded microparticles of PLGA 75:25 , E) loaded microparticles of PLGA 50:50

Table 20 Particle size of antigen-loaded PLGA microparticles

Polymer	Concentration (%)	Antigen added (ug)	Sonicate (output)	Particle size (um)			Uniformity
				D(v,0.1)	D(v,0.5)	D(v,0.9)	
DL-PLA	1.5	250	9	2.35	20.37	81.07	1.627E+00
			12	2.12	13.29	106.08	2.722E+00
			15	3.09	8.81	18.80	5.479E-01
		500	9	2.18	20.55	104.40	1.599E+00
			12	4.54	13.40	29.01	7.691E-01
			15	2.05	8.92	21.78	6.753E-01
		750	9	2.37	20.99	83.40	1.351E+00
			12	5.15	13.97	29.64	7.004E-01
			15	2.64	9.04	20.75	6.166E-01
	3	750	9	3.43	49.03	275.47	1.641E+00
			12	6.10	27.36	105.09	1.353E+00
			15	2.29	12.08	62.09	1.835E+00
	5	750	9	4.49	93.90	273.89	8.832E-01
			12	2.67	44.33	133.79	9.527E-01
			15	3.01	14.67	39.88	7.825E-01
PLGA 85:15	1.5	250	9	3.71	16.62	83.99	1.392E+00
			12	3.75	12.33	25.03	6.206E-01
			15	0.86	8.60	44.44	1.403E+00
		500	9	1.88	17.49	53.80	1.899E+00
			12	2.03	12.45	70.81	2.017E+00
			15	3.89	8.76	21.91	6.219E-01
		750	9	2.11	19.20	73.45	1.418E+00
			12	2.38	12.68	69.18	2.164E+00
			15	3.64	8.88	32.76	1.240E+00
	3	750	9	2.10	46.73	261.32	1.710E+00
			12	2.15	25.06	87.07	1.071E+00
			15	5.78	11.19	19.33	3.753E-01
	5	750	9	4.52	83.80	255.43	8.962E-01
			12	2.16	33.41	150.76	1.388E+00
			15	4.54	13.51	26.73	6.434E-01

Table 20 Particle size of antigen-loaded PLGA microparticles (cont.)

Polymer	Concentration (%)	Antigen added (ug)	Sonicate (output)	Particle size (um)			Uniformity
				D(v,0.1)	D(v,0.5)	D(v,0.9)	
PLGA 75:25	1.5	250	9	3.01	16.11	64.49	1.374E+00
			12	3.49	11.71	25.66	8.435E-01
			15	0.08	8.18	42.29	1.408E+00
		500	9	3.31	16.29	57.98	1.009E+00
			12	2.23	11.80	56.19	1.670E+00
			15	1.72	8.47	27.83	1.409E+00
		750	9	3.67	16.46	81.52	1.363E+00
			12	4.16	12.02	26.63	9.907E-01
			15	1.82	8.52	51.28	2.466E+00
	3	750	9	3.91	38.52	112.10	9.823E-01
			12	2.67	24.73	137.35	1.677E+00
			15	1.30	10.35	37.16	1.193E+00
	5	750	9	2.84	70.48	237.78	1.054E+00
			12	3.82	32.89	108.43	9.695E-01
			15	4.14	11.66	23.19	6.712E-01
PLGA 50:50	1.5	250	9	2.86	15.09	60.16	1.522E 00
			12	2.65	9.12	21.30	6.254E-01
			15	1.24	4.61	12.13	7.469E-01
		500	9	4.18	15.16	32.68	7.850E-01
			12	1.10	9.32	45.82	1.328E+00
			15	1.35	5.77	36.75	2.078E+00
		750	9	3.51	15.42	61.20	1.162E+00
			12	4.90	9.77	16.89	3.816E-01
			15	1.36	5.80	37.99	2.158E+00
	3	750	9	3.67	36.26	126.98	1.081E+00
			12	6.14	22.77	114.18	1.472E+00
			15	3.15	8.99	19.18	5.487E-01
	5	750	9	4.89	66.89	204.22	9.116E-01
			12	6.13	27.44	105.74	1.249E+00
			15	1.35	10.52	37.44	1.162E+00

concentration of 3% and 1.5%, respectively. However, the effect of the amount of antigen added in the process was not clearly observed. The microparticles composed of 250, 500 and 750 μg of antigen were merely different in size.

1.1.3 Antigen content and % entrapment efficiency

The antigen content and percent entrapment efficiency of PLGA micro - particles are shown in Table 21 - 24 for DL-PLA, PLGA 85:15, PLGA 75:25 and PLGA 50:50, respectively. The antigen content and entrapment efficiency were affected by many factors such as amount of antigen added in formulations, copolymer composition, polymer concentration, and shearing force used in preparation of primary emulsion. The entrapment efficiency was ranging between 40-70%. PLGA 50:50 had the greatest encapsulation efficiency followed by PLGA 75:25, PLGA 85:15 and DL-PLA, respectively. The increment in concentration of polymer decreased antigen content but increased in entrapment efficiency. In addition, both antigen content and entrapment efficiency were improved by increasing the amount of antigen added in the manufacturing process from 250 to 750 μg. However, the increment in the sonicating force did not sufficiently change the antigen content and entrapment efficiency in microparticles.

1.1.4 In vitro antigen release

In vitro release profiles from PLGA microparticles incubated in PBS pH 7.4 over 15 days are depicted in figures 30 – 41 for 1.5, 3 and 5% of DL-PLA, PLGA 85:15, PLGA 75:25 and PLGA 50:50 respectively. The release profiles showed similar pattern for each polymer. The antigen release was found to be most rapid from PLGA 50:50, approximately of 60% antigen release within 15 days following by those from microparticles prepared with PLGA 75:25, PLGA 85 :15 and DL-PLA, respectively. The lowest antigen release was observed from microparticles prepared with 5 % DL-

Table 21 Antigen content and entrapment efficiency from DL-PLA microparticles

Polymer	Concentration (%)	Antigen added (ug)	Sonicate (output)	Antigen loading (ug)	(SD)	Entrapment efficiency (%)	Antigen content (ug/100 mg sample)
DL-PLA	1.5	250	9	92.82	(5.28)	37.13	73.15
			12	95.82	(4.85)	38.33	76.60
			15	101.76	(5.11)	40.70	83.75
		500	9	217.01	(9.90)	43.40	179.79
			12	222.14	(9.22)	44.43	178.71
			15	230.11	(10.39)	46.02	187.69
		750	9	384.93	(18.74)	51.32	320.24
			12	392.54	(19.25)	52.34	318.37
			15	401.15	(21.05)	53.49	327.73
	3	250	9	106.41	(6.14)	42.56	41.58
			12	107.95	(5.91)	43.18	42.79
			15	109.26	(6.29)	43.70	41.96
		500	9	239.00	(12.20)	47.80	97.51
			12	239.61	(16.91)	47.92	95.31
			15	244.89	(14.80)	48.98	93.19
		750	9	412.51	(21.25)	55.00	174.42
			12	419.98	(19.89)	56.00	175.87
			15	425.19	(19.62)	56.69	174.04
	5	250	9	117.70	(7.17)	47.08	28.06
			12	119.40	(9.41)	47.76	28.25
			15	120.59	(5.92)	48.24	28.06
		500	9	261.42	(16.04)	52.28	61.63
			12	264.94	(14.92)	52.99	62.06
			15	268.76	(18.70)	53.75	63.93
		750	9	450.80	(22.80)	60.11	105.50
			12	455.55	(25.55)	60.74	107.04
			15	457.25	(25.27)	60.97	109.73

Table 22 Antigen content and entrapment efficiency from PLGA 85:15 microparticles

Polymer	Concentration (%)	Antigen added (ug)	Sonicate (output)	Antigen loading (ug)	(SD)	Entrapment efficiency (%)	Antigen content (ug/100 mg sample)
PLGA 85:15	1.5	250	9	100.93	(7.35)	40.37	82.33
			12	102.06	(6.62)	40.82	80.74
			15	103.12	(8.78)	41.25	85.93
		500	9	237.82	(17.33)	47.56	188.45
			12	239.22	(19.10)	47.84	190.01
			15	240.12	(20.21)	48.02	191.94
		750	9	412.46	(24.60)	54.99	337.53
			12	413.31	(23.33)	55.11	341.30
			15	417.03	(27.14)	55.60	347.24
	3	250	9	114.12	(5.21)	45.65	48.44
			12	114.23	(8.30)	45.69	49.30
			15	115.41	(6.15)	46.16	51.54
		500	9	250.06	(11.60)	50.01	110.50
			12	252.25	(12.55)	50.45	109.58
			15	252.78	(11.86)	50.56	113.46
		750	9	433.76	(23.40)	57.83	184.58
			12	442.41	(24.29)	58.99	190.12
			15	446.47	(26.60)	59.53	190.88
	5	250	9	127.75	(7.55)	51.10	29.13
			12	128.39	(7.14)	51.36	30.56
			15	129.35	(8.80)	51.74	29.40
		500	9	279.66	(12.65)	55.93	64.19
			12	281.82	(11.46)	56.36	63.57
			15	288.96	(15.60)	57.79	66.75
		750	9	475.02	(25.20)	63.34	109.25
			12	479.04	(24.38)	63.87	111.64
			15	482.36	(28.76)	64.31	114.25

Table 23 Antigen content and entrapment efficiency from PLGA 75:25 microparticles

Polymer	Concentration (%)	Antigen added (ug)	Sonicate (output)	Antigen loading (ug)	(SD)	Entrapment efficiency (%)	Antigen content (ug/100 mg sample)
PLGA 75:25	1.5	250	9	111.62	(6.22)	44.65	93.09
			12	111.87	(8.08)	44.75	92.30
			15	112.31	(6.84)	44.92	90.86
		500	9	254.89	(14.42)	50.98	212.76
			12	257.52	(12.95)	51.50	213.00
			15	258.66	(16.19)	51.73	218.28
		750	9	450.00	(25.00)	60.00	389.95
			12	453.94	(23.80)	60.53	373.00
			15	456.93	(25.90)	60.92	386.57
	3	250	9	118.46	(6.85)	47.38	48.19
			12	120.95	(8.20)	48.38	50.25
			15	121.26	(7.55)	48.50	50.91
		500	9	282.05	(15.59)	56.41	116.98
			12	281.87	(14.70)	56.37	118.19
			15	284.42	(14.20)	56.88	113.00
		750	9	467.81	(28.74)	62.37	186.38
			12	471.65	(21.48)	62.89	20.70
			15	476.25	(26.50)	63.50	206.35
	5	250	9	132.36	(6.93)	52.94	29.31
			12	134.39	(6.03)	53.76	31.47
			15	137.49	(5.90)	55.00	31.97
		500	9	291.61	(11.26)	58.32	67.86
			12	293.62	(13.92)	58.72	64.25
			15	298.19	(11.80)	59.64	69.25
		750	9	496.31	(26.75)	66.17	114.59
			12	499.49	(24.90)	66.60	118.00
			15	500.2	(25.55)	66.69	113.68

Table 24 Antigen content and entrapment efficiency from PLGA 50:50 microparticles

Polymer	Concentration (%)	Antigen added (ug)	Sonicate output)+D12	Antigen loading (ug)	(SD)	Entrapment efficiency (%)	Antigen content (ug/100 mg sample)
PLGA 50:50	1.5	250	9	119.63	(9.10)	47.85	92.30
			12	122.57	(8.75)	49.03	99.25
			15	123.71	(7.67)	49.48	98.49
		500	9	272.61	(15.70)	54.52	233.00
			12	273.62	(13.82)	54.72	223.18
			15	275.02	(15.00)	55.00	237.70
		750	9	460.07	(27.70)	61.34	375.88
			12	464.44	(29.40)	61.93	384.15
			15	465.75	(28.84)	62.10	384.92
	3	250	9	129.71	(7.15)	51.88	56.69
			12	131.75	(5.70)	52.70	59.13
			15	131.44	(5.84)	52.58	55.56
		500	9	293.85	(14.48)	58.77	124.25
			12	296.11	(14.10)	59.22	129.25
			15	298.92	(16.22)	59.78	126.88
		750	9	496.55	(30.65)	66.21	209.25
			12	498.02	(28.29)	66.40	215.50
			15	498.5	(28.05)	66.47	217.78
	5	250	9	146.95	(7.71)	58.78	34.18
			12	147.07	(6.70)	58.83	32.01
			15	151.63	(6.35)	60.65	35.54
		500	9	331.33	(17.80)	66.27	76.75
			12	338.56	(15.53)	67.71	81.42
			15	340.87	(14.65)	68.17	78.23
		750	9	537.28	(27.82)	71.64	121.75
			12	537.02	(28.20)	71.60	118.00
			15	543.67	(24.95)	72.49	124.13

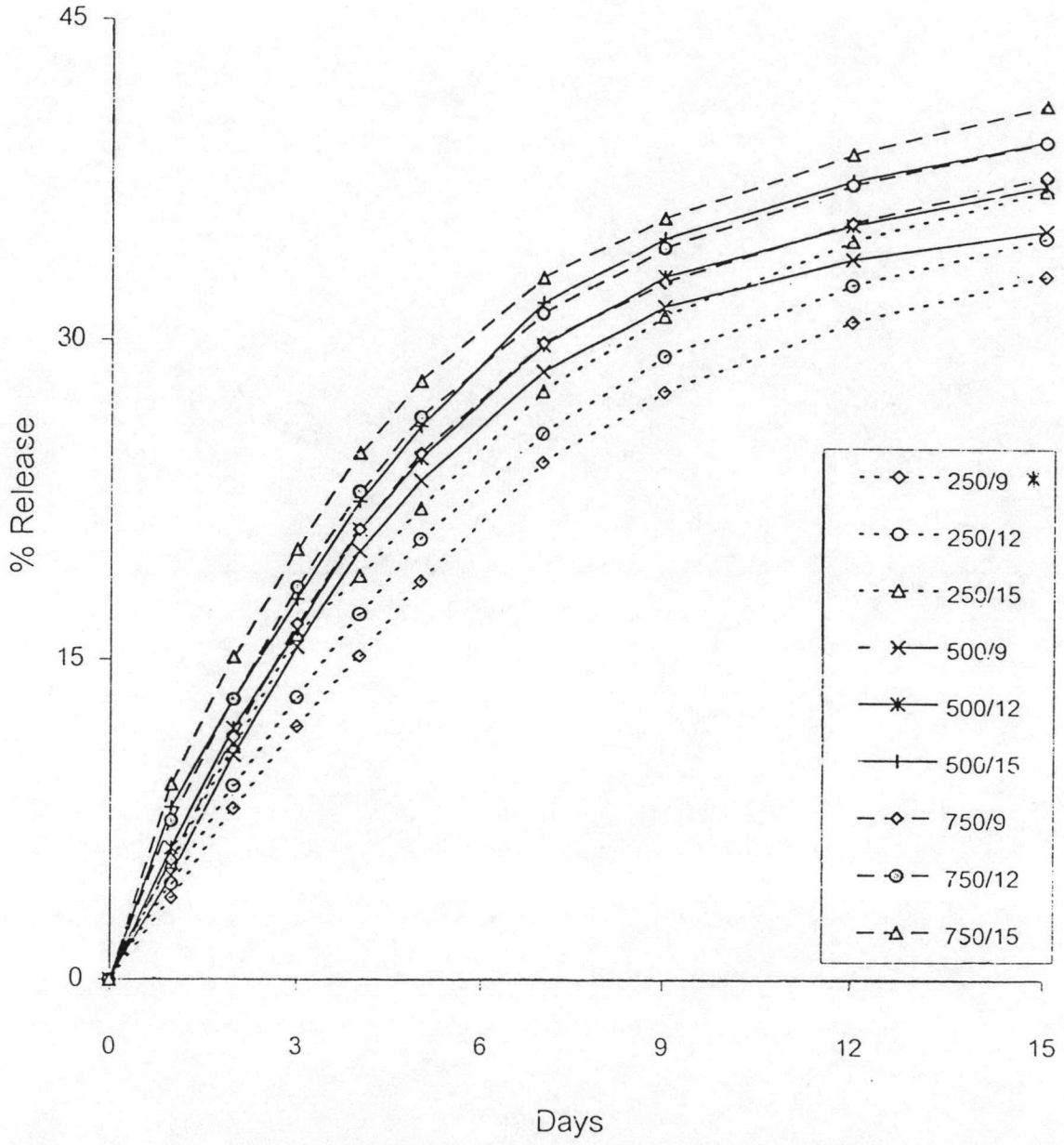


Figure 30 In vitro antigen release profiles of microparticles prepared with 1.5 % DL - PLA polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

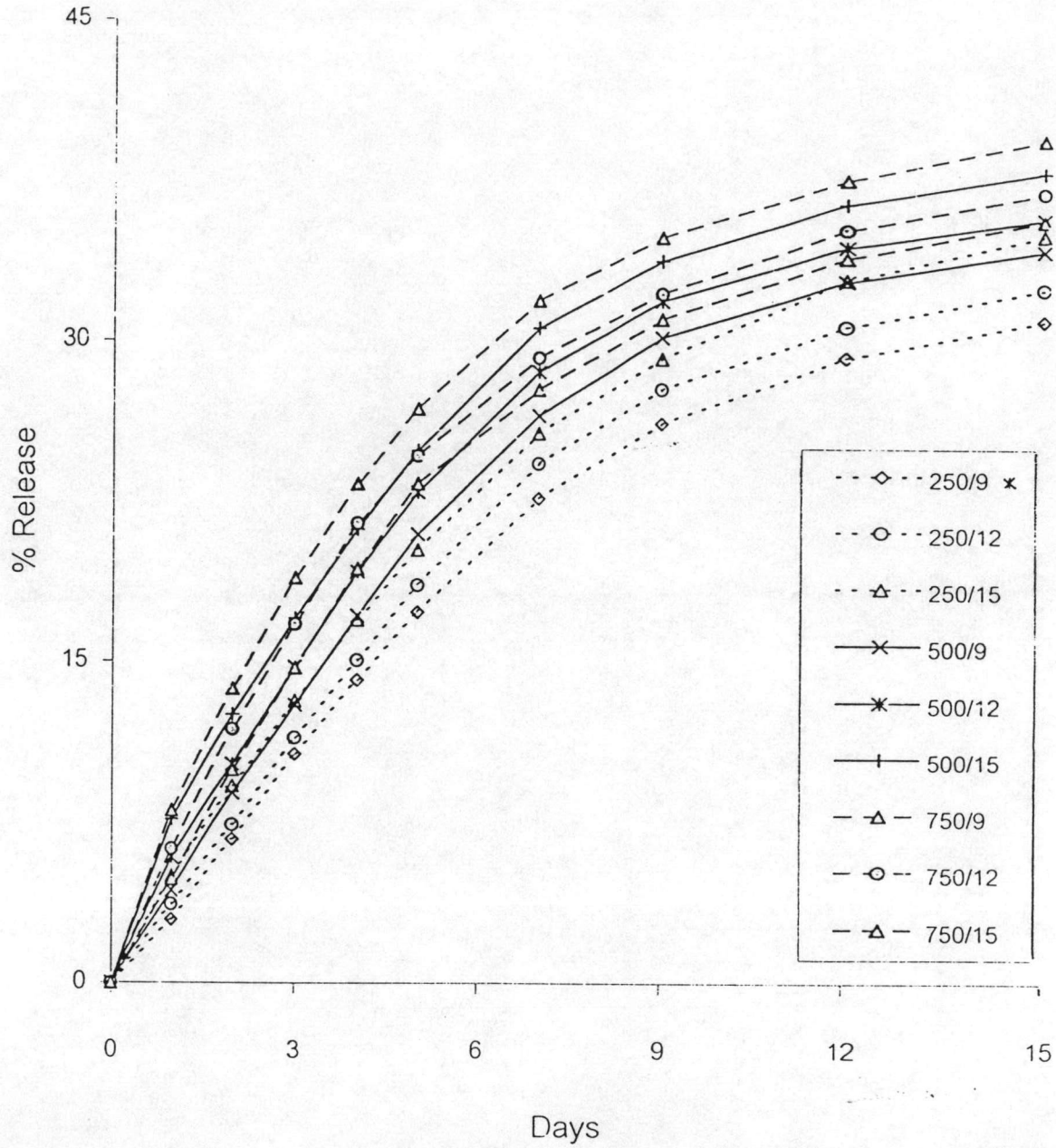


Figure 31 In vitro antigen release profiles of microparticles prepared with 3 % DL - PLA polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

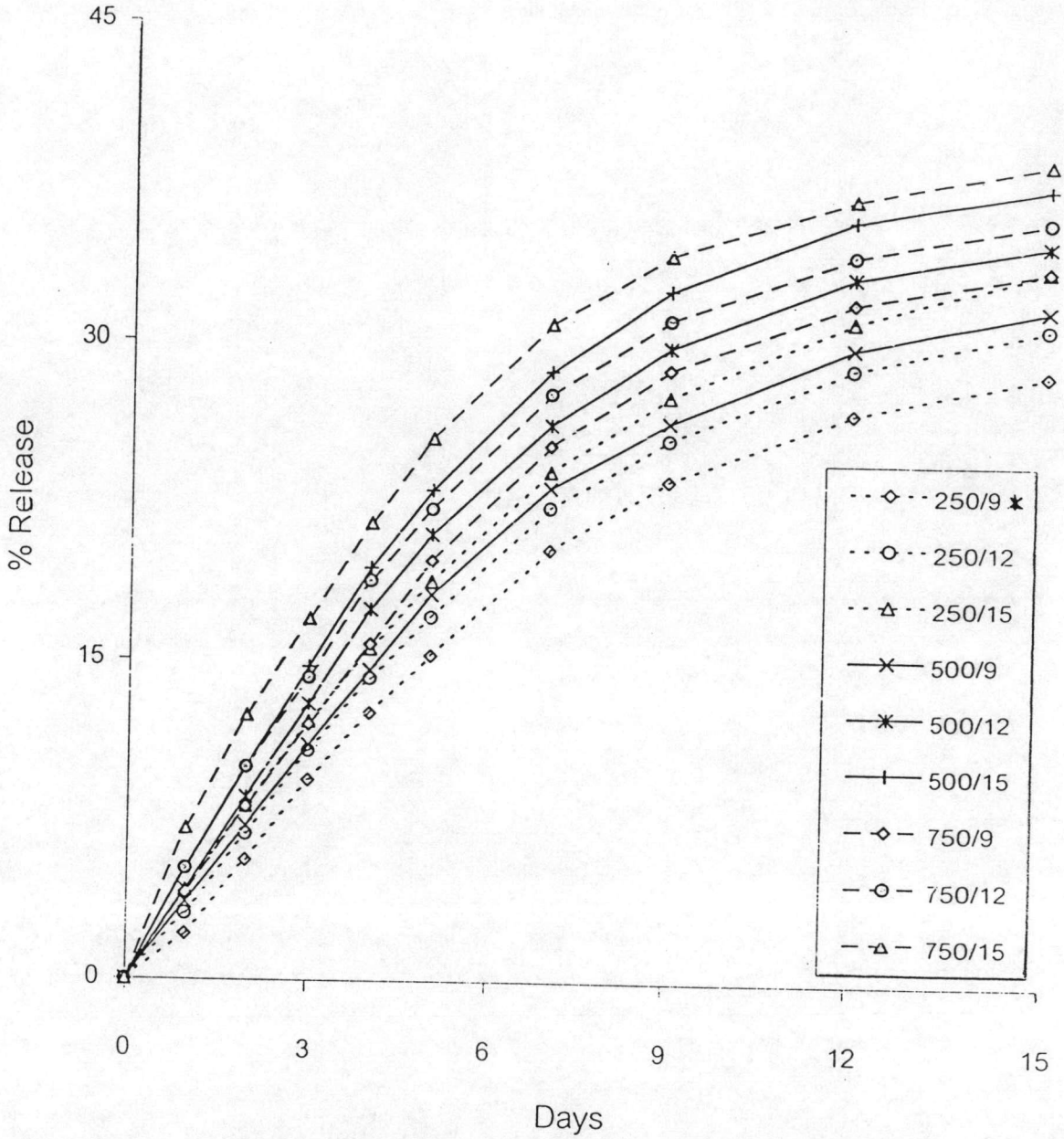


Figure 32 In vitro antigen release profiles of microparticles prepared with 5 % DL - PLA polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

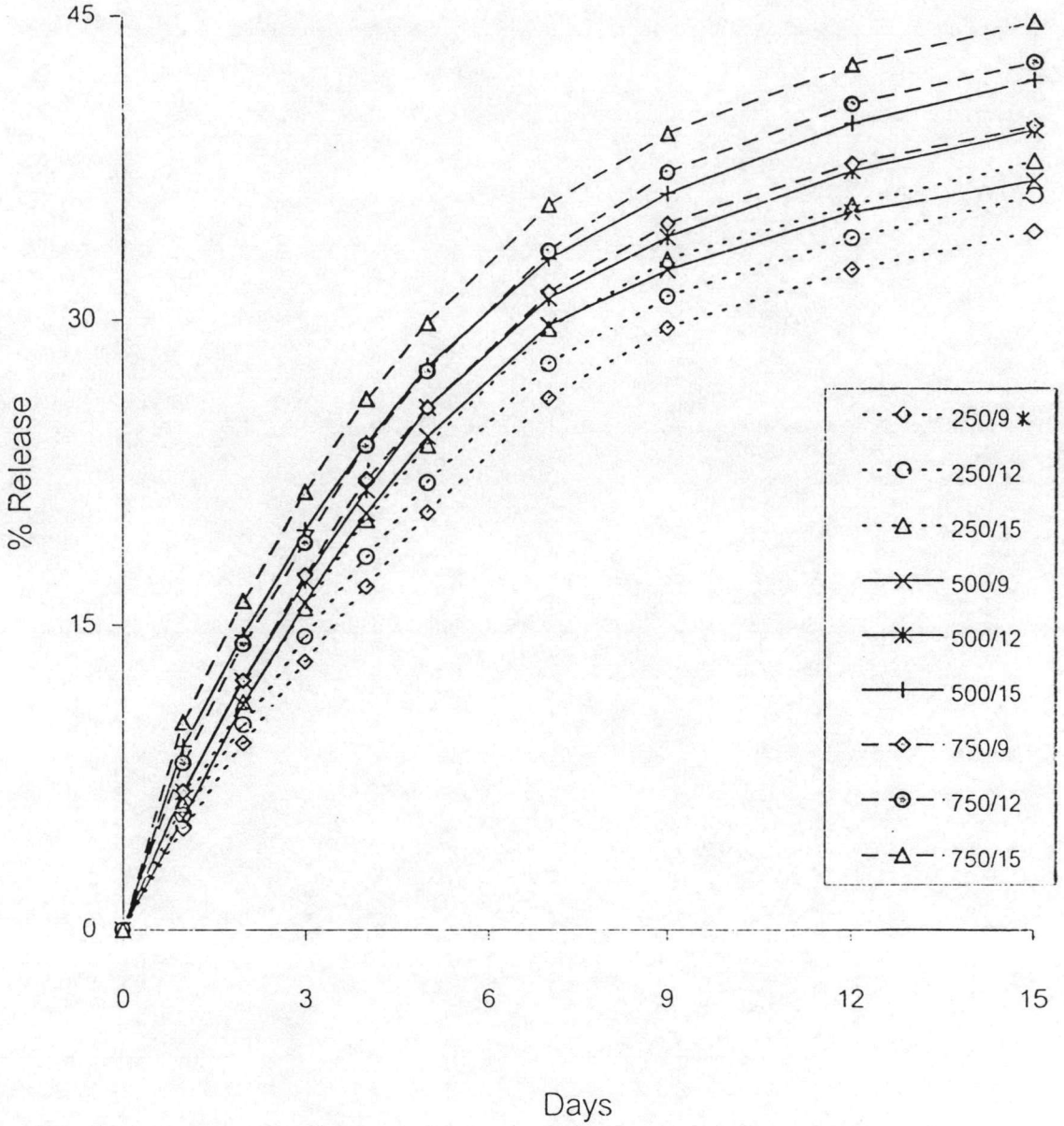


Figure 33 In vitro antigen release profiles of microparticles prepared with 1.5 % PLGA 85:15 polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

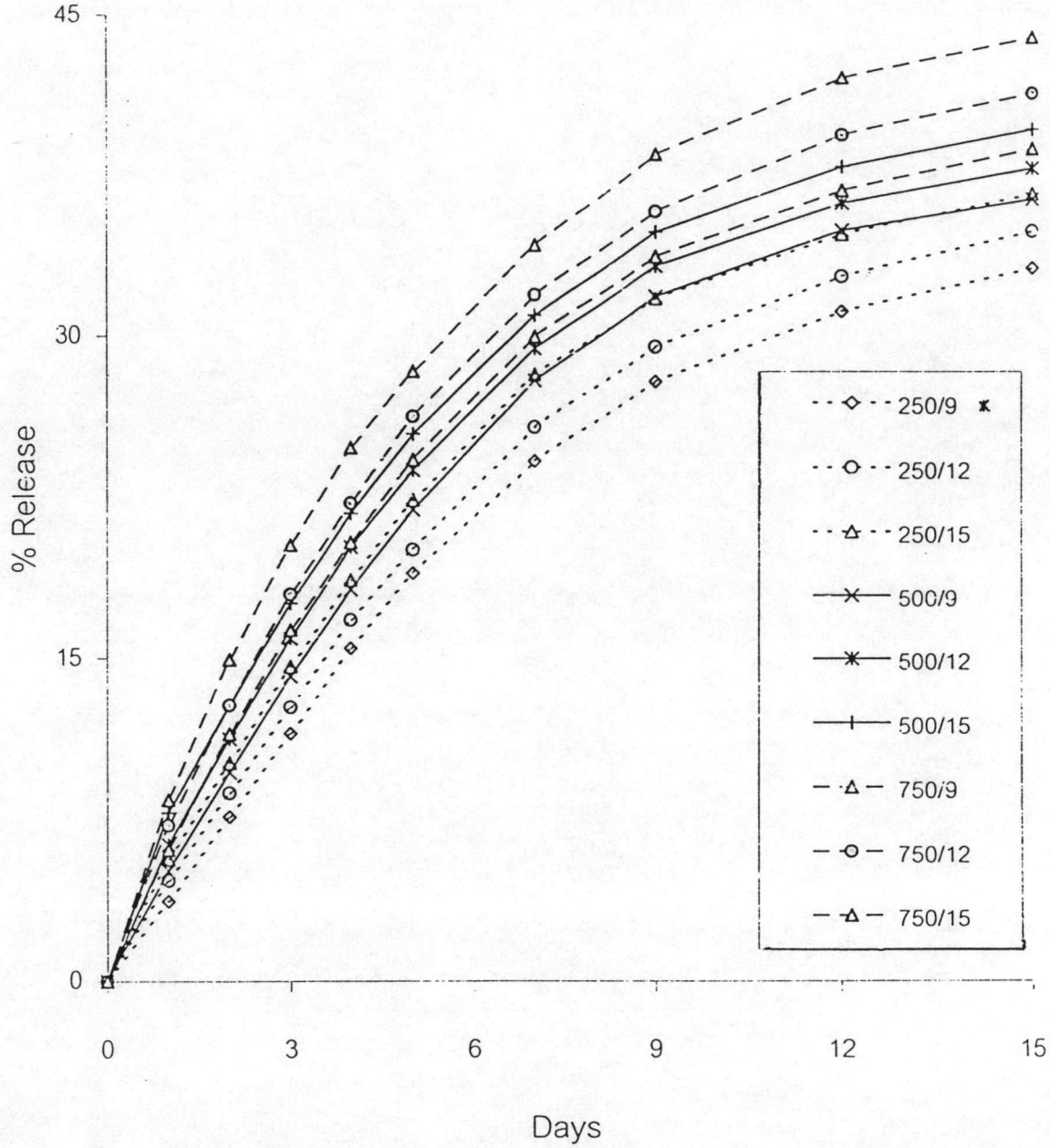


Figure 34 In vitro antigen release profiles of microparticles prepared with 3 % PLGA 85:15 polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

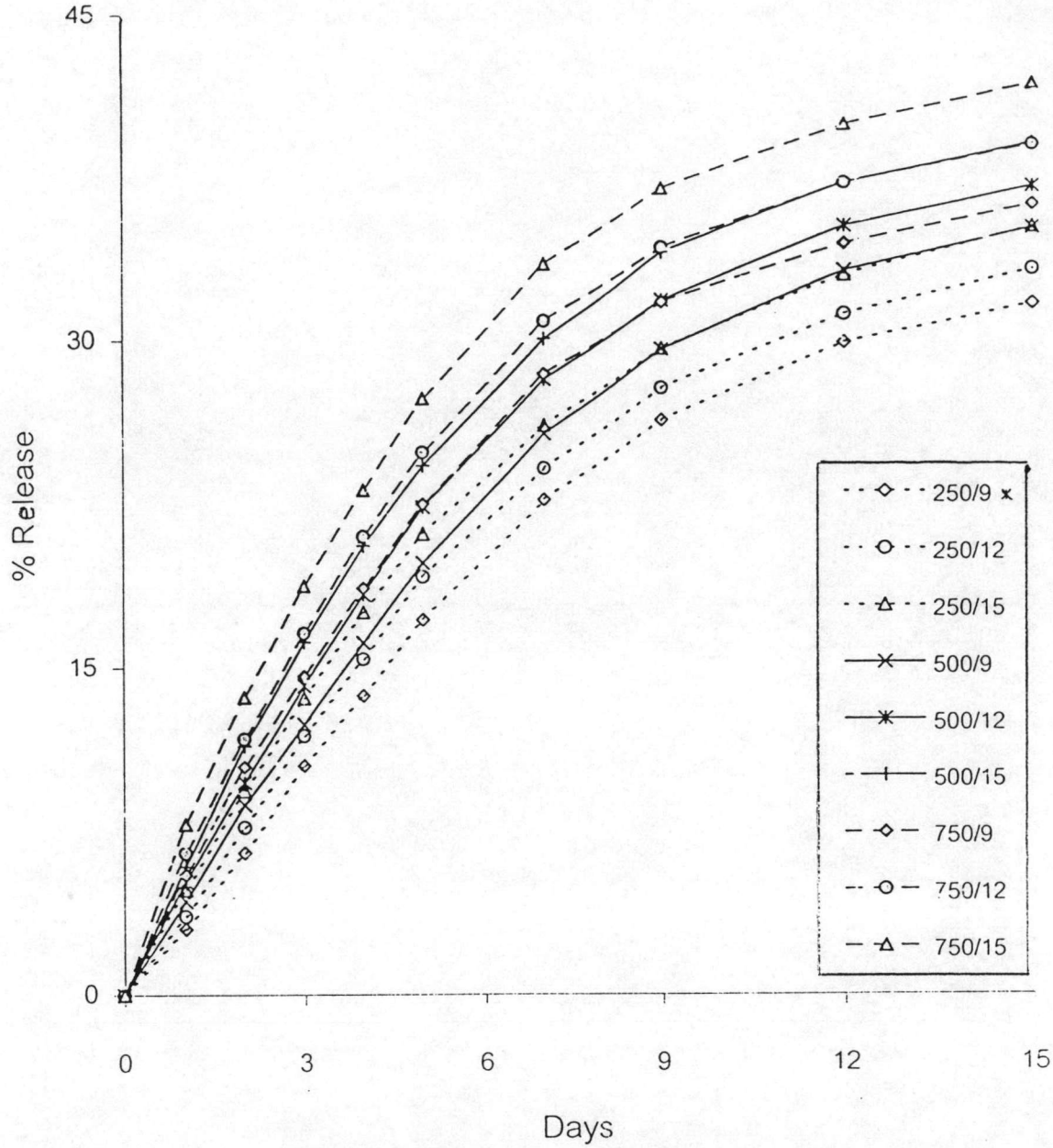


Figure 35 In vitro antigen release profiles of microparticles prepared with 5 % PLGA 85:15 polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

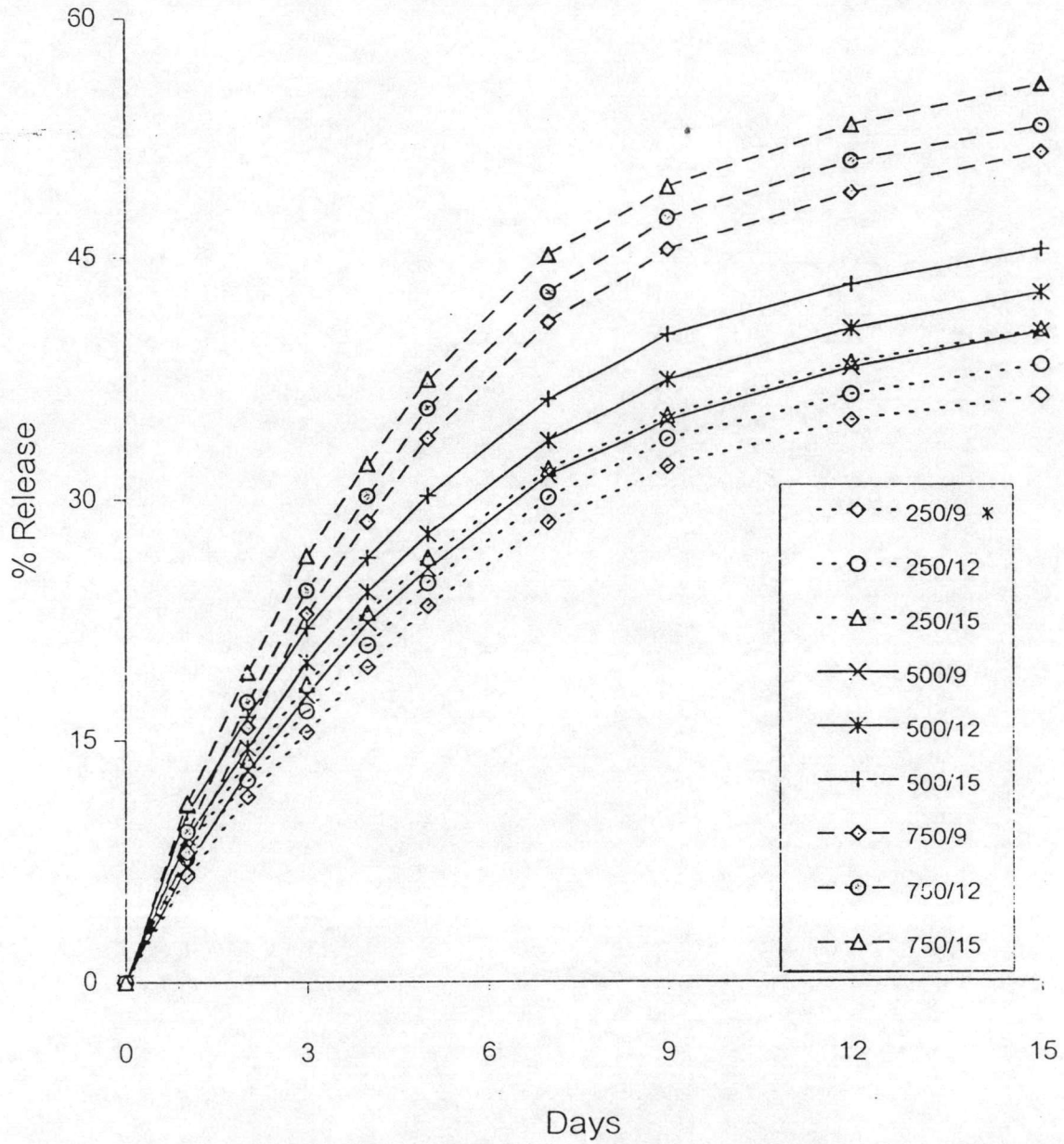


Figure 36 In vitro antigen release profiles of microparticles prepared with 1.5 % PLGA 75:25 polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

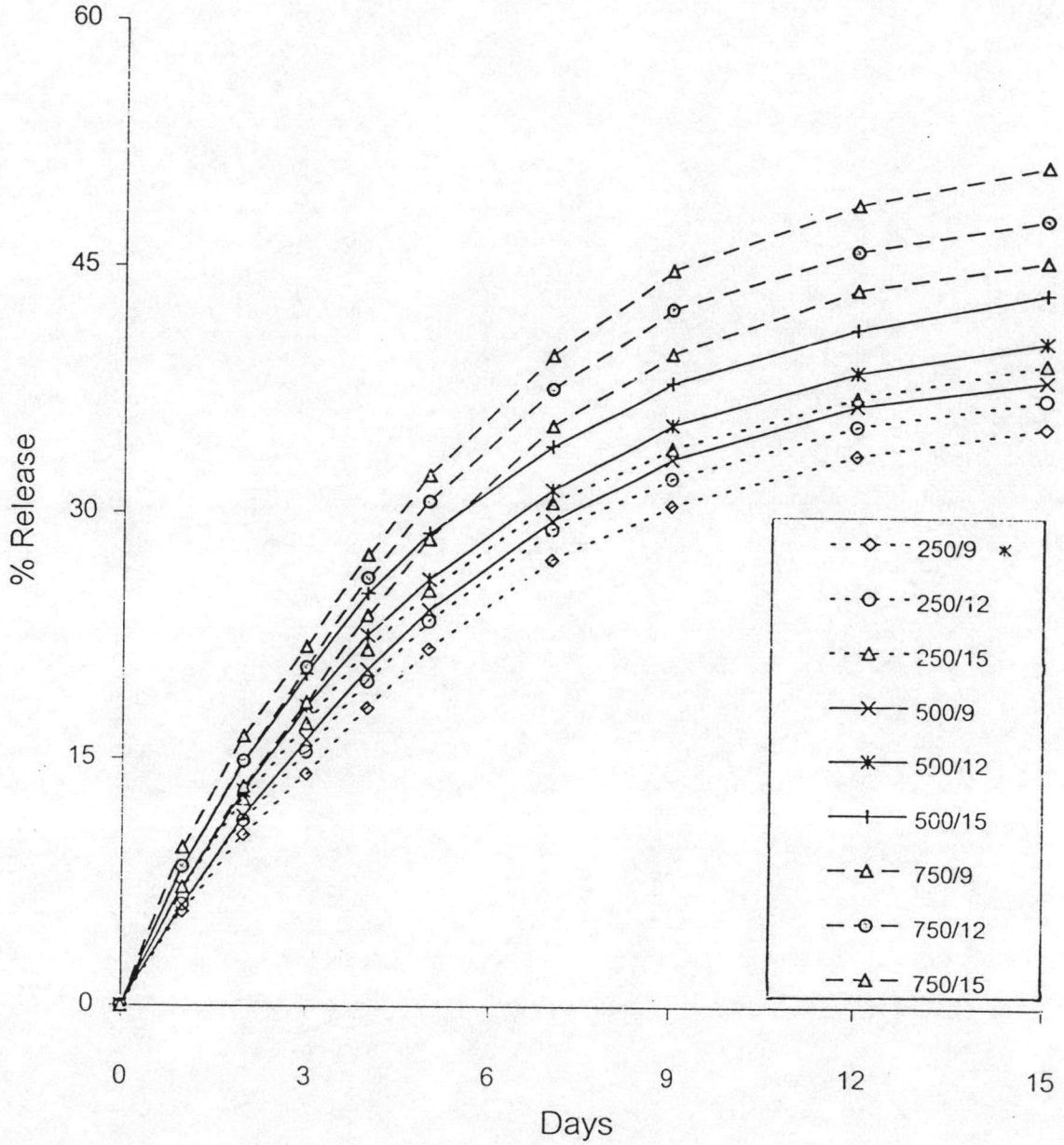


Figure 37 In vitro antigen release profiles of microparticles prepared with 3 % PLGA 75:25 polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

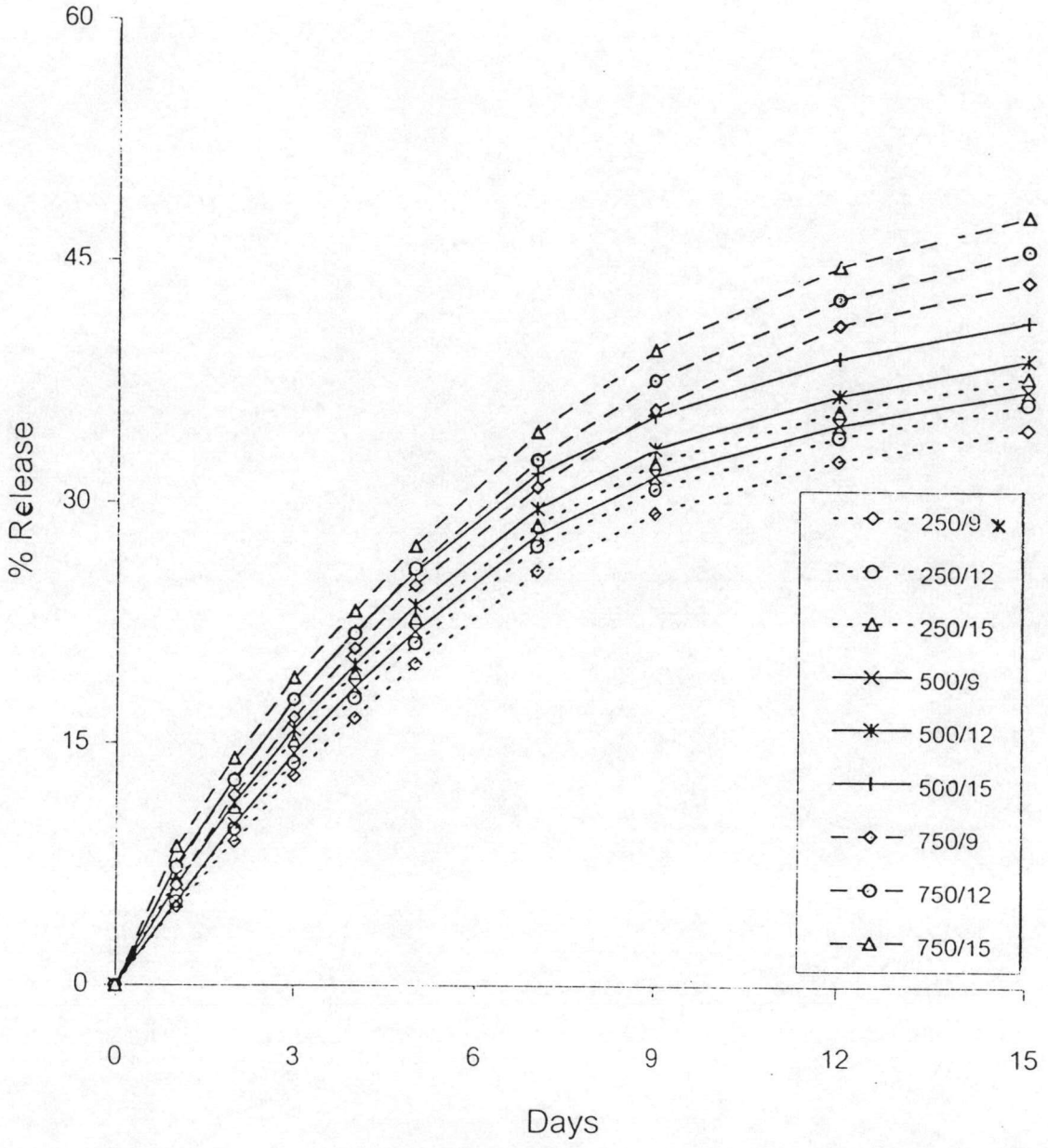


Figure 38 In vitro antigen release profiles of microparticles prepared with 5 % PLGA 75:25 polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

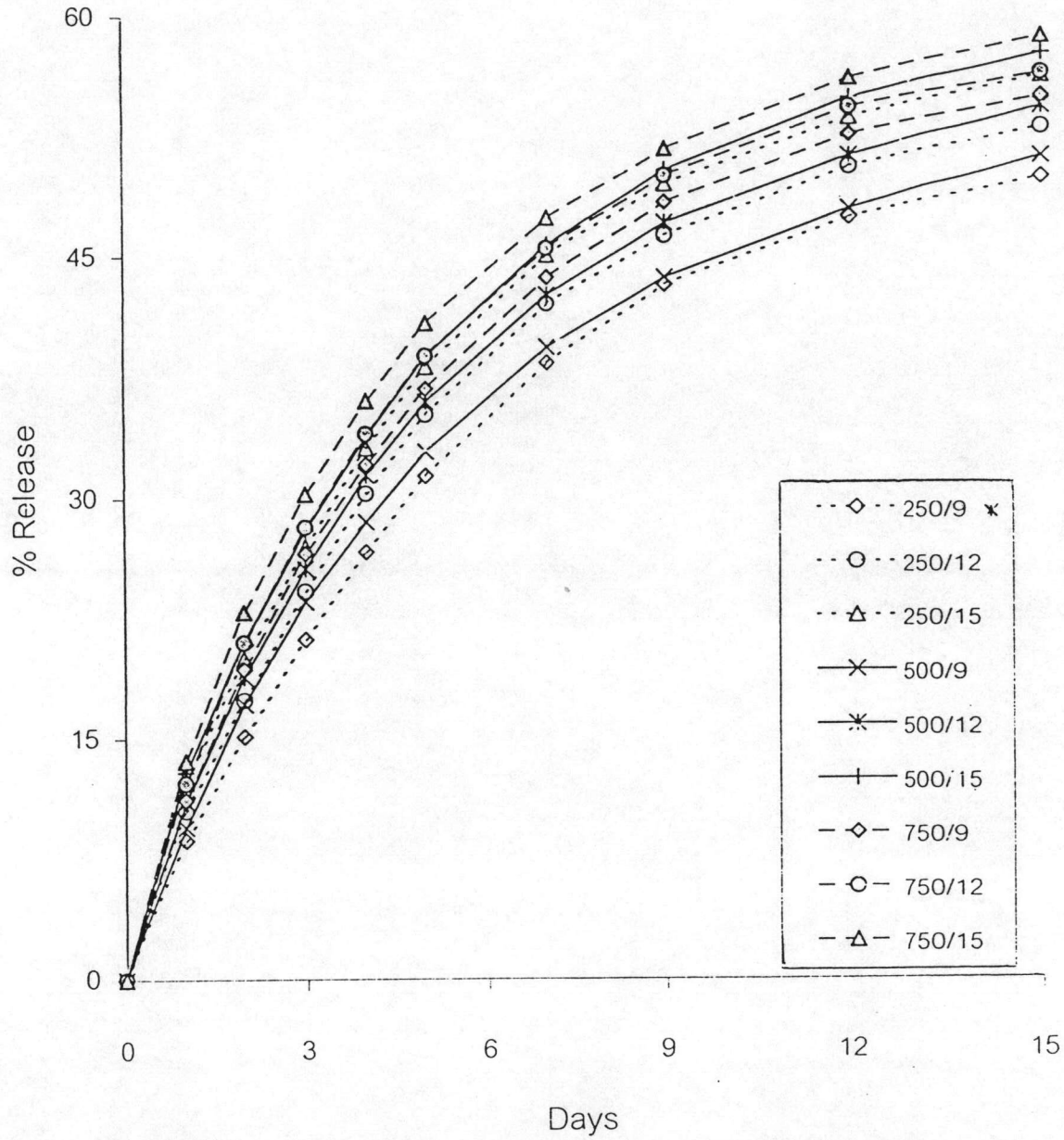


Figure 39 In vitro antigen release profiles of microparticles prepared with 1.5 % PLGA 50:50 polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

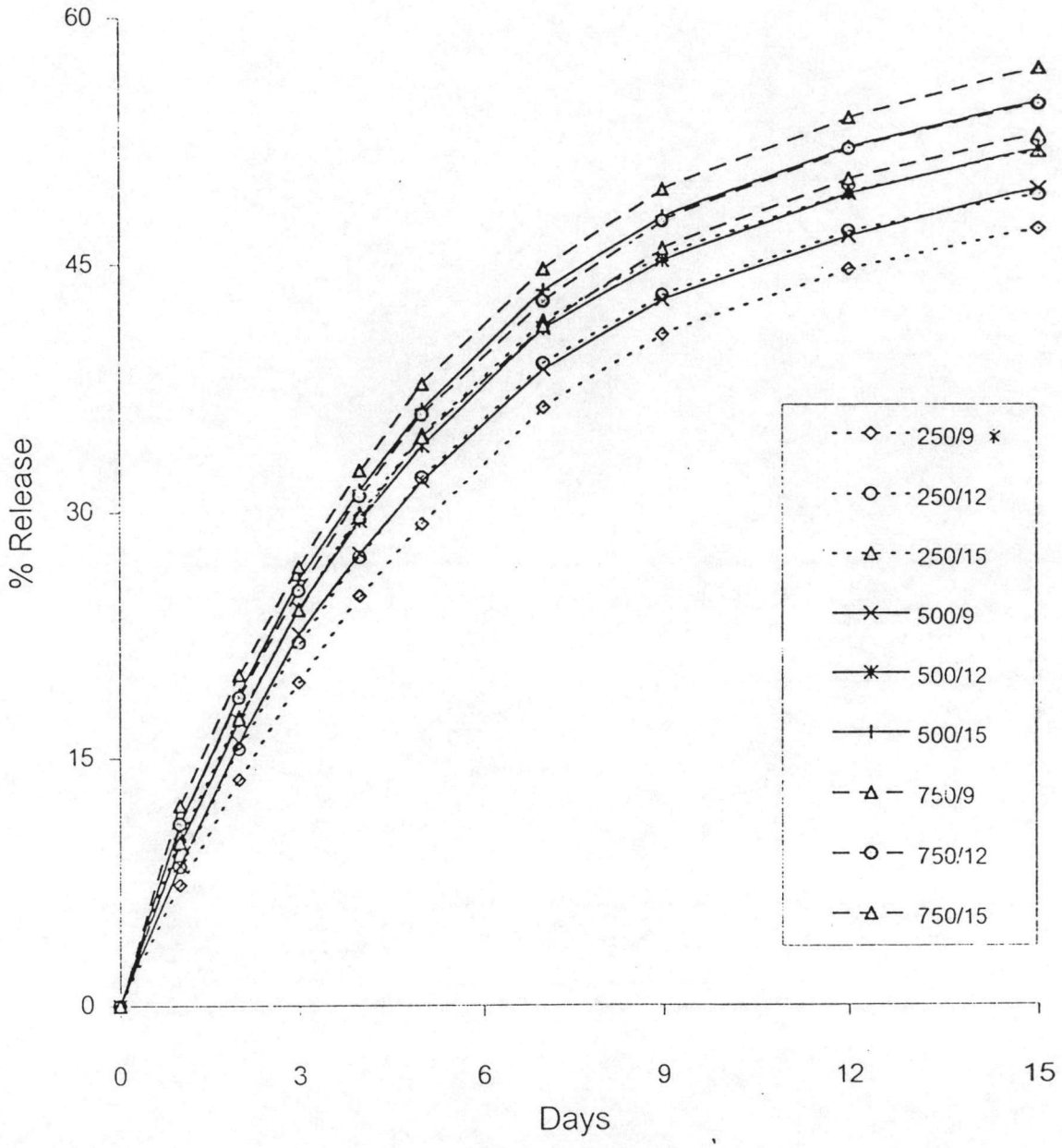


Figure 40 In vitro antigen release profiles of microparticles prepared with 3 % PLGA 50:50 polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

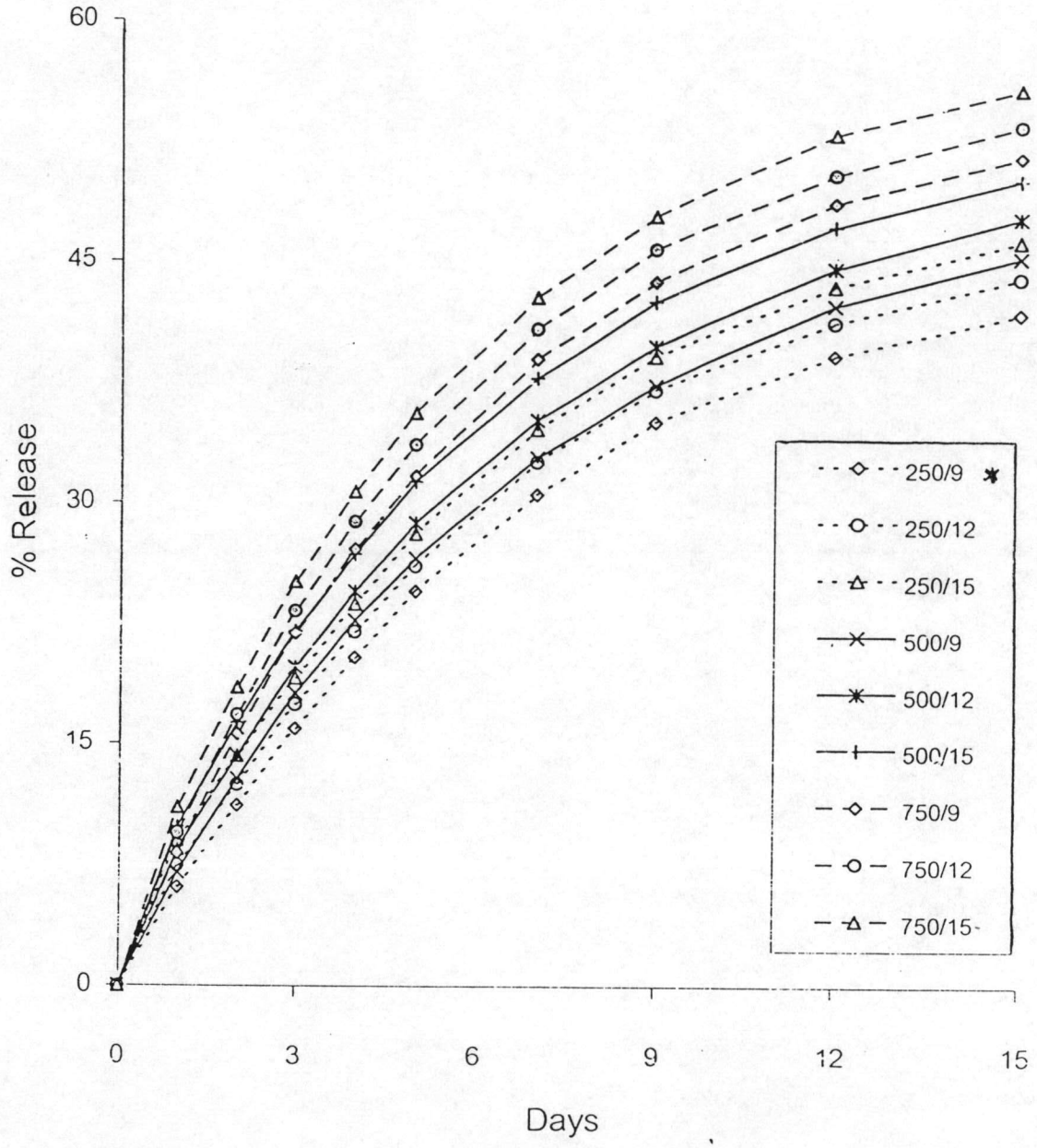


Figure 41 In vitro antigen release profiles of microparticles prepared with 5 % PLGA 50:50 polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

PLA which exhibited an approximately of 30% release. Additionally, polymer concentration affected the antigen release. Higher polymer concentration evidently produced slower release of antigen. Further, particle size influenced on antigen release from microparticles. Smaller size of microparticles apparently exhibited higher release of antigen.

1.1.5 Levels of residual solvent

The residual dichloromethane from 500 mg of microparticulate samples produced by w/o/w solvent evaporation up to 8 hours is denoted in Table 25. All tested samples had dichloromethane residue in the range of 5-15 ppm. This range was in the restriction of the United State Pharmacopoeia, 75 ppm (USP 23). Therefore, it was acceptable to use for injection in animal models and humans.

1.1.6 Antigen integrity

The integrity of antigen determined by SDS-PAGE clearly showed three bands of protein as in figure 42. The protein bands had molecular weights approximately of 30, 35 and 50 kDa. There was no additional band present from antigen encapsulated in microparticles, comparison to pure JE antigen. Therefore, no aggregation of protein and no smaller molecular weight fragments was led. Consequently, it was concluded that PLGA microparticles produced from w/o/w emulsion solvent evaporation which involved in an organic solvent such as dichloromethane still maintained the integrity of antigen.

1.1.7 Polymer degradation

The degradation of unloaded PLGA microparticles was analysed in term of molecular weight distribution using GPC. The molecular weight of polymer at different time of degradation is summarized in Table 26. The degradation profiles

Table 25 Level of residual dichloromethane in PLGA microparticles (n = 3)

Formulation	DCM content (ppm)	SD
P1	8.76	0.06
P2	9.04	0.11
P3	8.40	0.09
P28	9.81	0.08
P29	9.38	0.12
P30	10.30	0.22
P55	10.54	0.14
P56	10.73	0.17
P57	11.17	0.24
P82	9.65	0.16
P83	10.10	0.23
P84	8.82	0.14

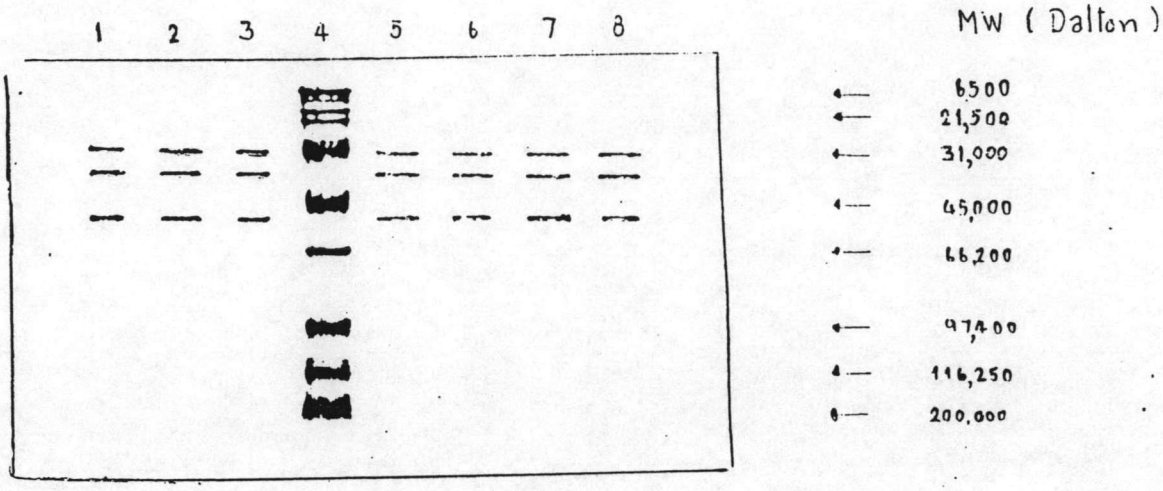


Figure 42 Structural integrity of JE antigen encapsulated in PLGA micro-particles ; JE antigen from DL-PLA : lane 5, PLGA 85:15 : lane 6, PLGA 75:25 : lane 7, PLGA 50:50 : lane 8, pure JE antigen : lane 1, 2,3 and protein standard marker : lane 4

Table 26 Molecular weight of PLGA polymers at different time of degradation

Time (weeks)	Results	Polymer			
		DL-PLA	PLGA 85:15	PLGA 75:25	PLGA 50:50
0	MW (Dalton)	76213	72997	67027	41312
	Polydispersity	2.117134	1.658187	2.106010	1.771585
2	MW (Dalton)	76043	72719	66781	39670
	Polydispersity	1.653324	1.791100	1.981207	1.692449
4	MW (Dalton)	75789	71952	65331	36627
	Polydispersity	1.822842	1.729852	1.598128	1.850329
6	MW (Dalton)	75528	71060	63504	32375
	Polydispersity	1.809821	1.845621	1.519140	1.834358
8	MW (Dalton)	75213	67705	58765	24189
	Polydispersity	1.936402	1.809084	1.739920	1.752271
10	MW (Dalton)	74642	63256	51028	14986
	Polydispersity	1.600258	1.955569	1.794887	1.774682
12	MW (Dalton)	73456	57630	39684	2307
	Polydispersity	1.455430	1.795471	1.808280	1.217281
16	MW (Dalton)	70180	43475	11631	**
	Polydispersity	1.636339	2.364588	1.773706	**

** data could not determine

analysed by GPC depicted in figure 43 indicated that molecular weight of all PLGA polymers decreased with time. However, the profile of decrease in molecular weight was different for each polymer. PLGA 50:50 microparticles showed the fastest degradation followed by PLGA 75:25, PLGA 85:15 and DL-PLA microparticles, respectively. In fact, the weight-average molecular weight of PLGA 50:50 decreased by 90% after incubation in PBS pH 7.4 for 3 months. Whereas the weight-average molecular weight of DL-PLA decreased only by 10% over the same period.

A typical scanning electron micrograph of microparticles at different stages of degradation is illustrated in figures 44-47. After appropriate time periods, very small pores were observed. The pore size enlarged and scattered all over the microparticles with time. However, DL-PLA microparticles did not loss their spherical shape along the degradation study. In contrast, PLGA 50:50 microparticles absolutely lost their spherical appearance over the same period.

1.2 Chitosan microparticles

1.2.1 Shape and surface morphology

The shape and surface morphology of microparticles prepared from LMW, MMW and HMW chitosan are depicted in figure 48. The Cryo-SEM micrographs illustrated that most microparticles were nonporous and spherical. However rough, uneven surface and some agglomeration were observed from chitosan microparticles of high molecular weight.

1.2.2 Particle size and size distribution

The mean particle size of antigen-loaded chitosan microparticles determined by laser particle size analyser were ranging from 6-10 μm. The particle size and size distribution of each formulation are summarized in Table 27. Particle size

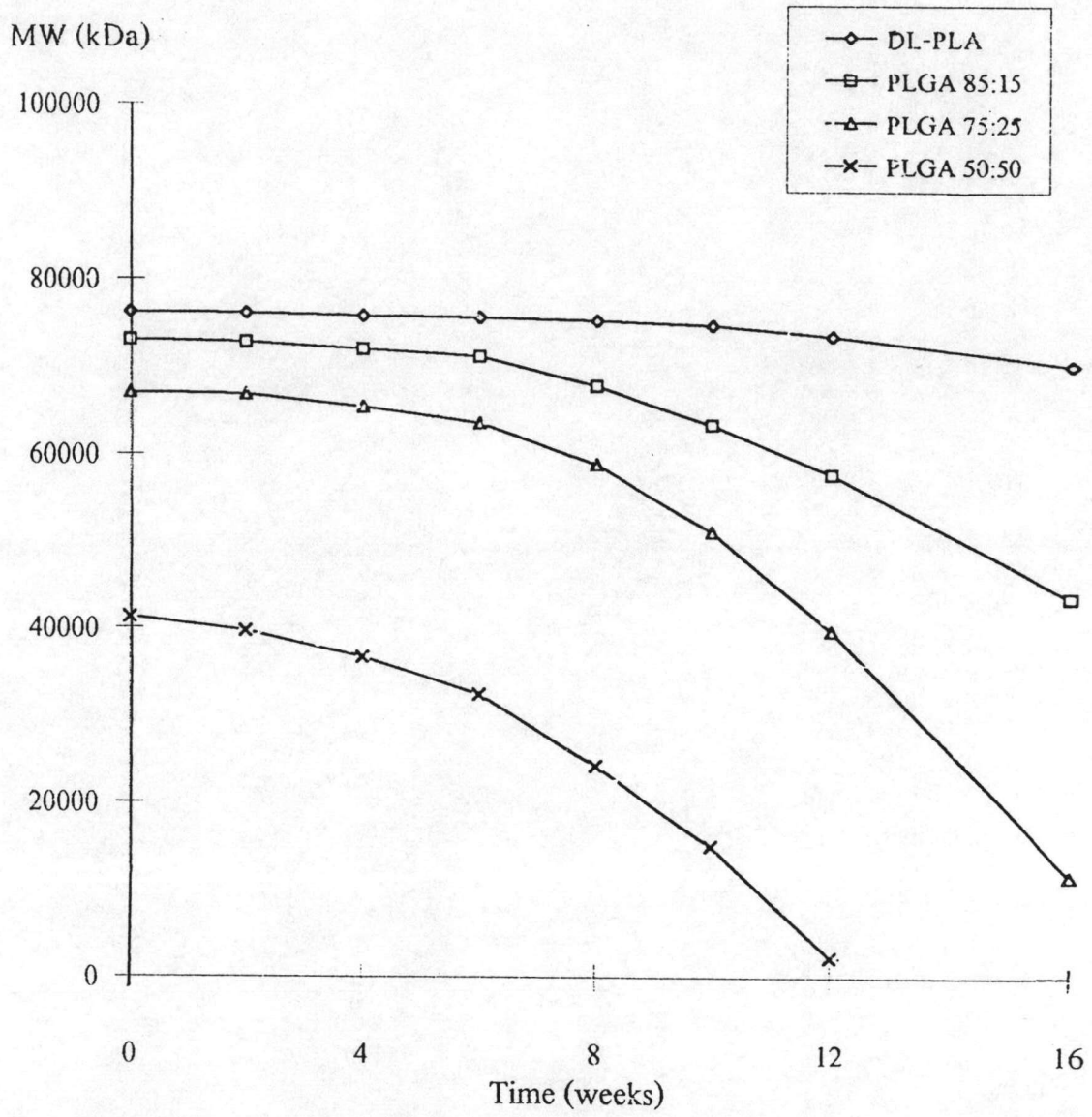


Figure 43 In vitro degradation profiles of unloaded PLGA microparticles incubated in PBS pH 7.4 at different time periods

A)

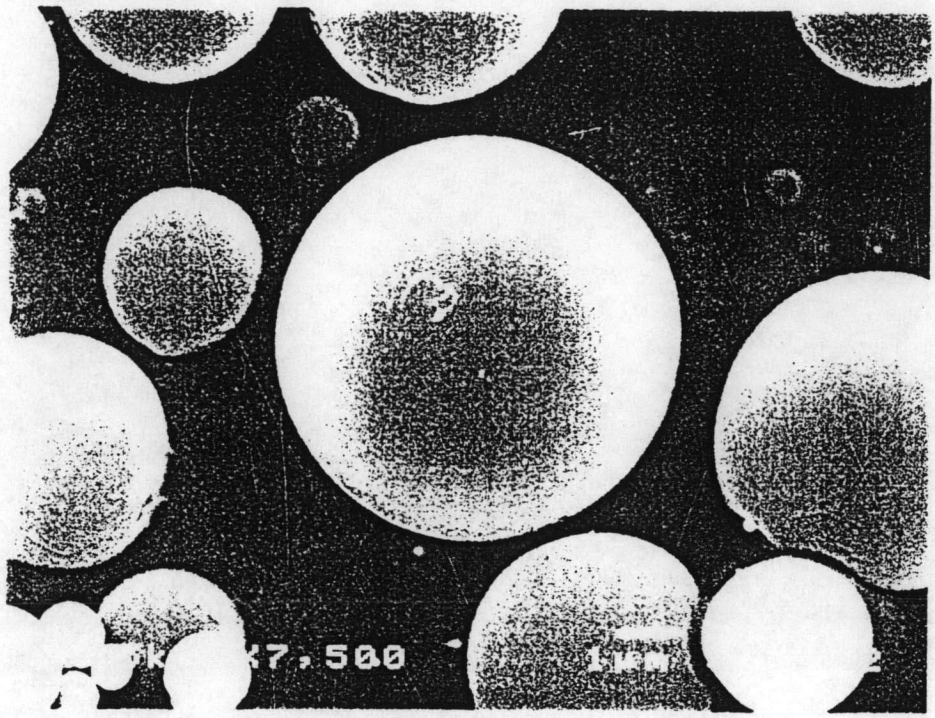


Figure 44 SEM micrographs of unloaded microparticles degeadation prepared with DL-PLA incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

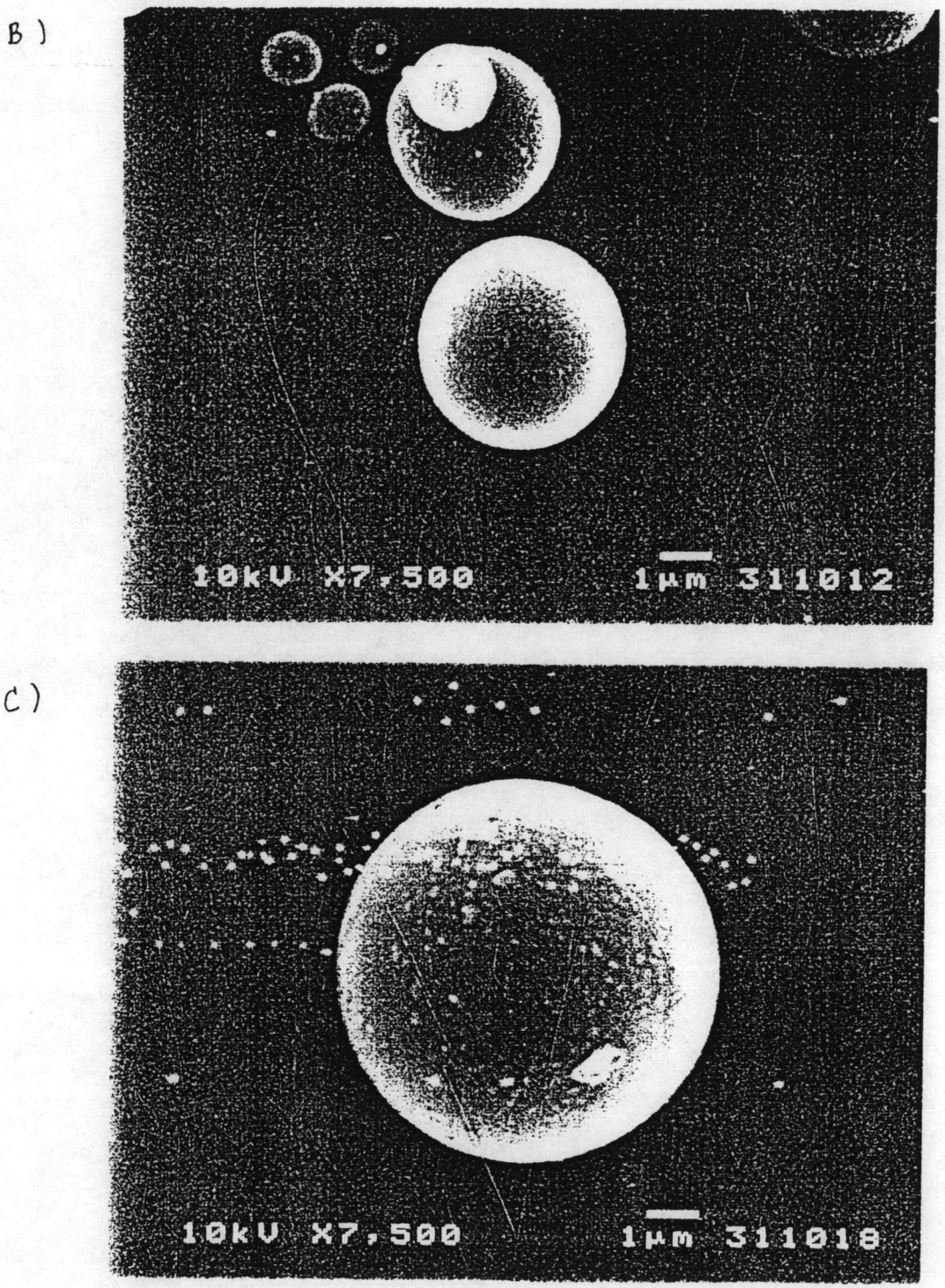


Figure 44 SEM micrographs of unloaded microparticles degeadation prepared with DL-PLA incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

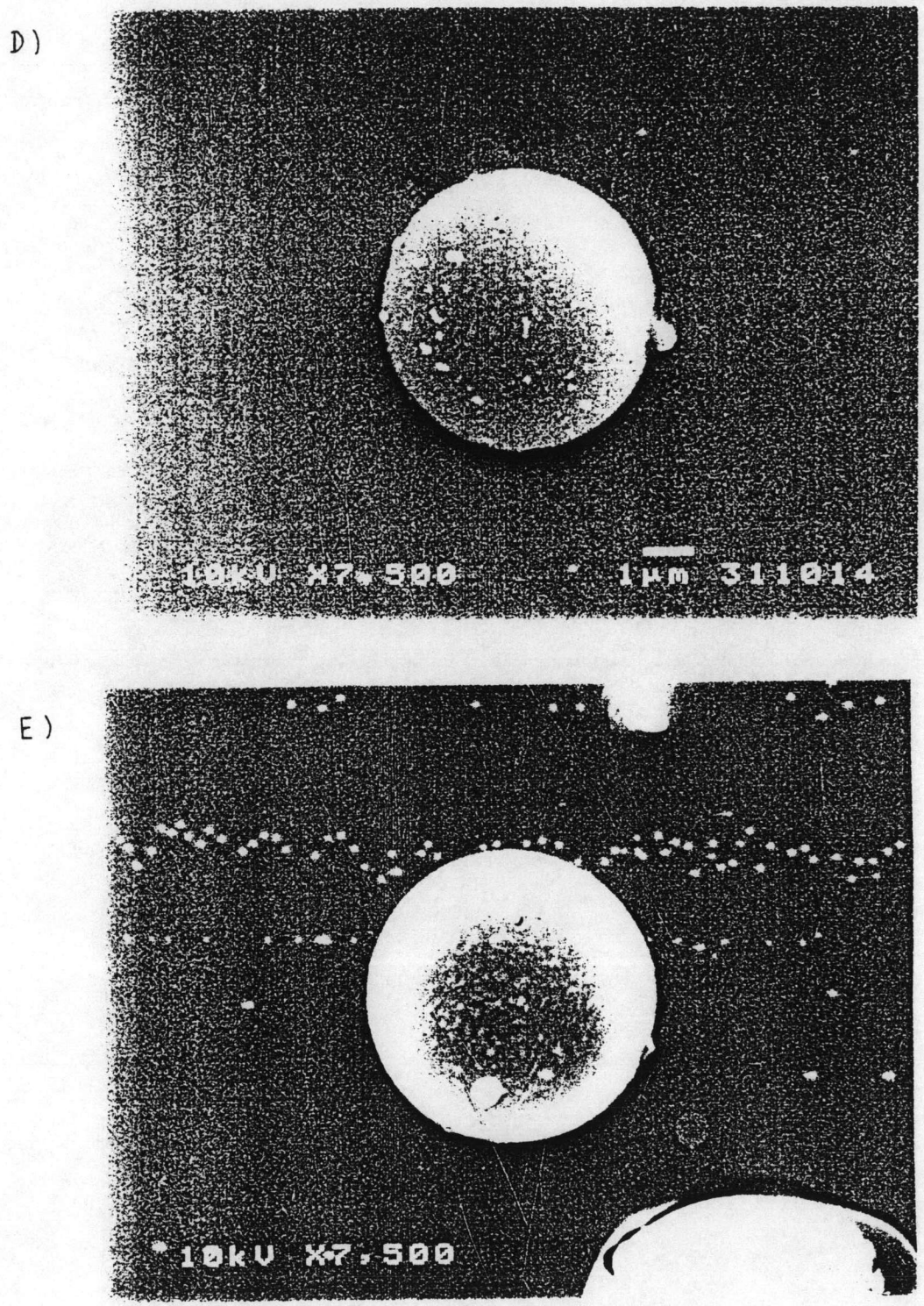


Figure 44 SEM micrographs of unloaded microparticles degeadation prepared with DL-PLA incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

A)

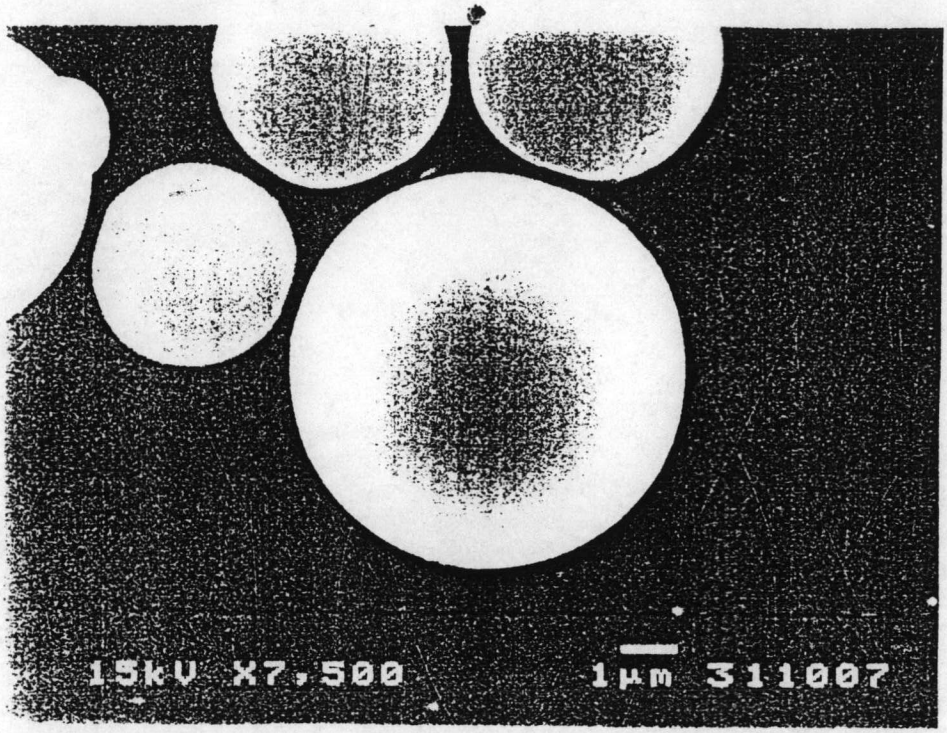


Figure 45 SEM micrographs of unloaded microparticles degeadation prepared with PLGA 85:15 incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

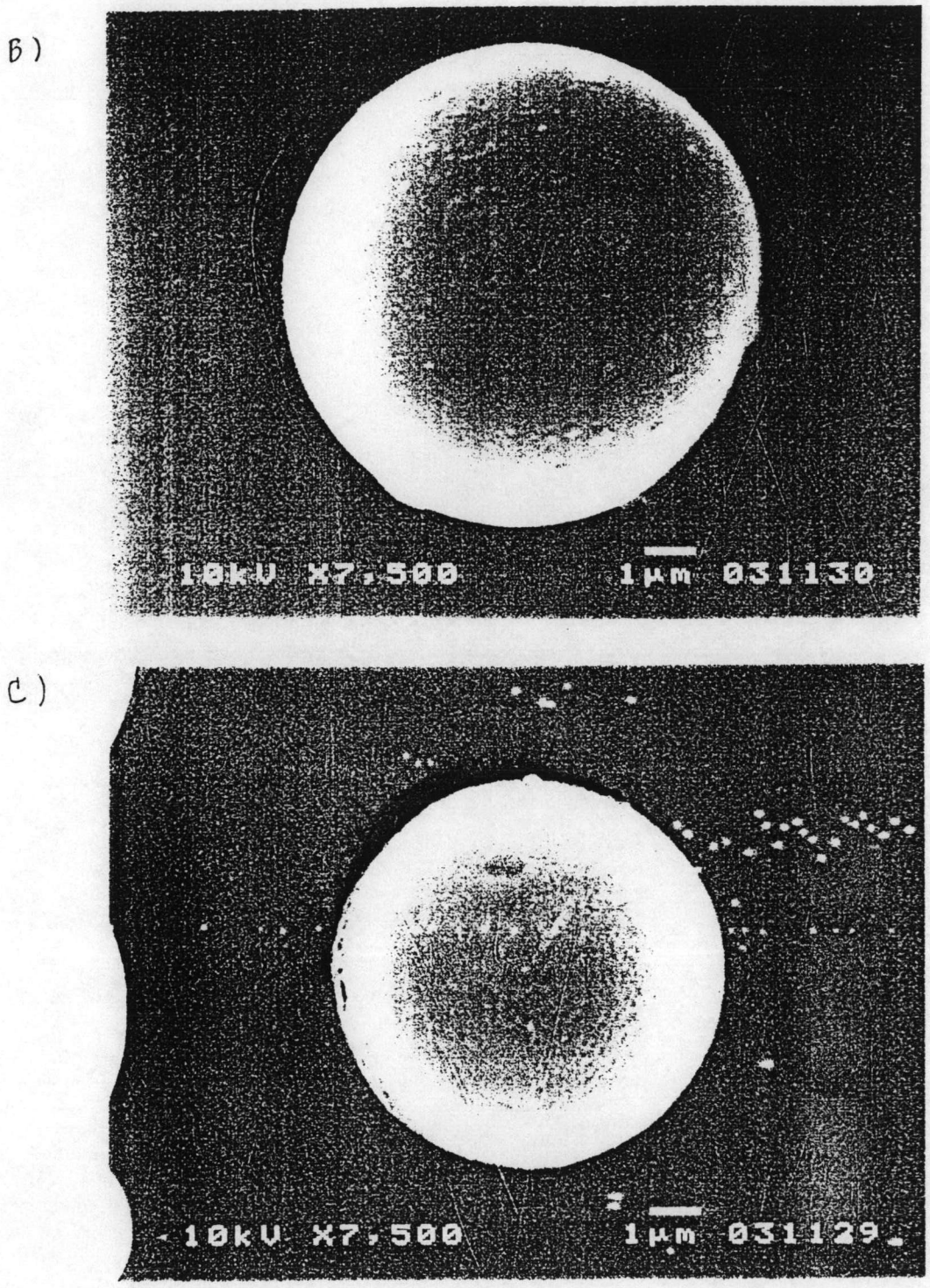


Figure 45 SEM micrographs of unloaded microparticles degeadation prepared with PLGA 85:15 incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

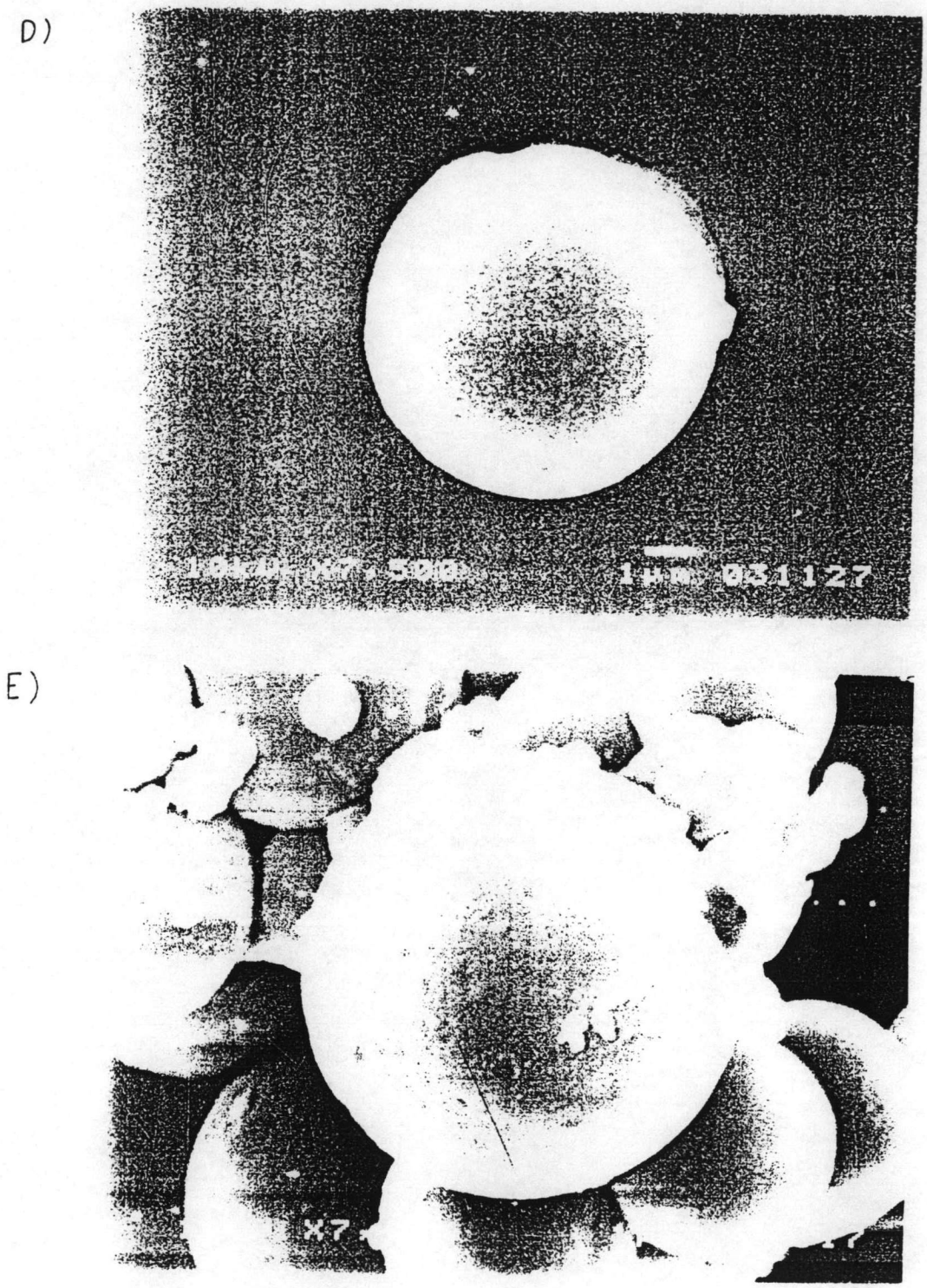


Figure 45 SEM micrographs of unloaded microparticles degeadation prepared with PLGA 85:15 incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

A)

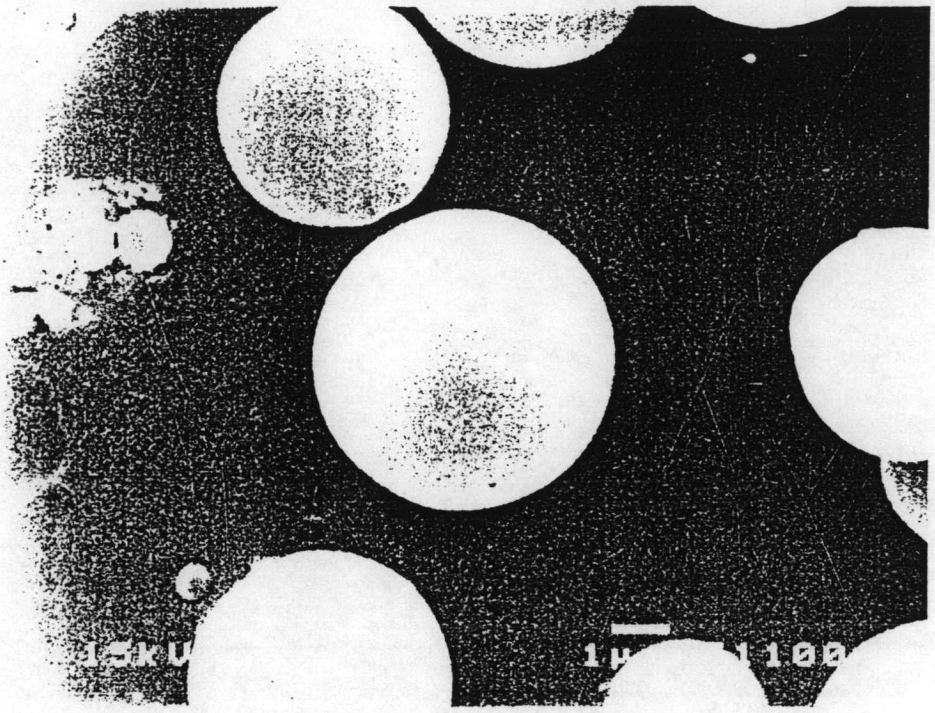


Figure 46 SEM micrographs of unloaded microparticles degeadation prepared with PLGA 75:25 incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

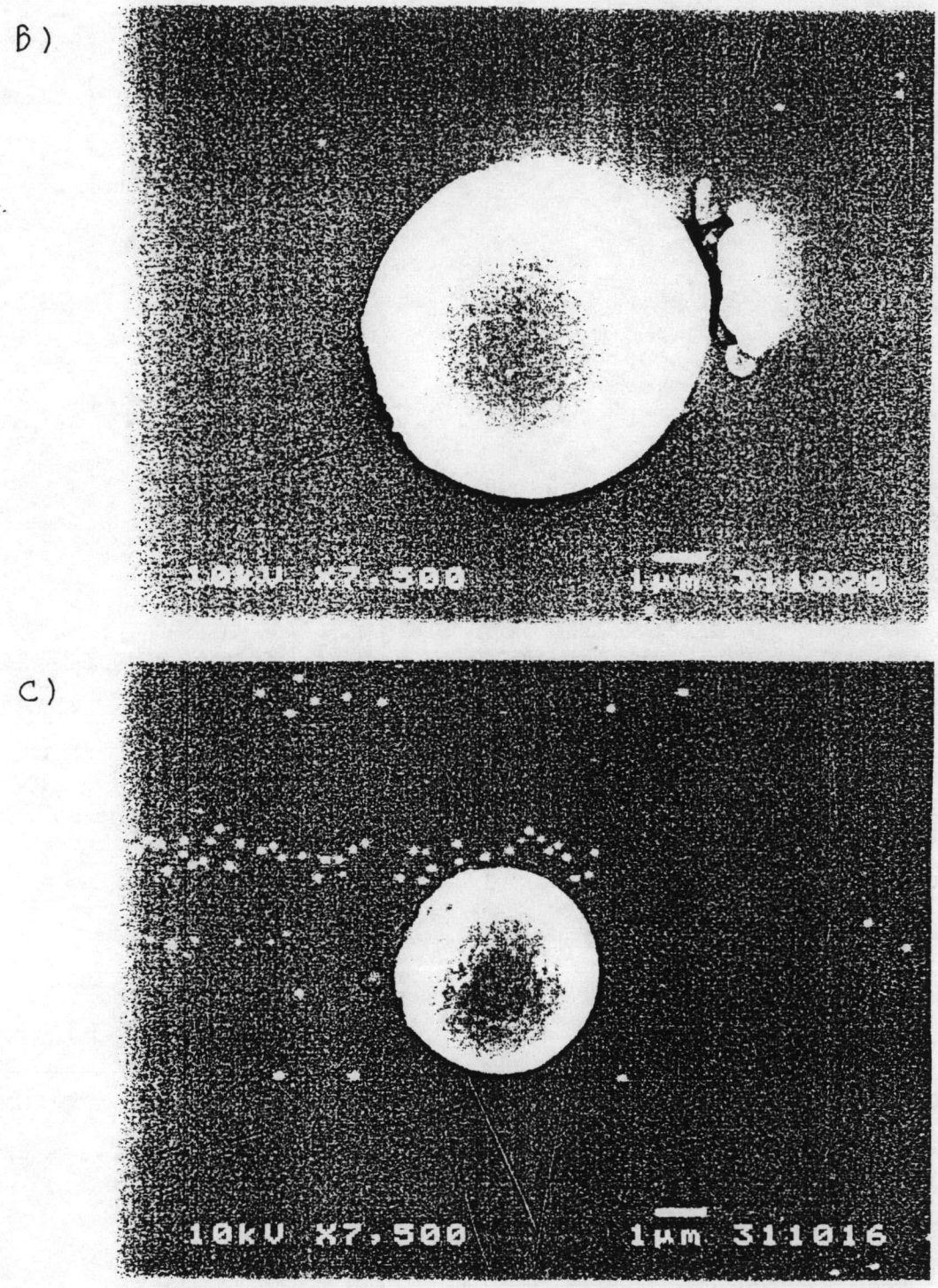


Figure 46 SEM micrographs of unloaded microparticles degeadation prepared with PLGA 75:25 incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

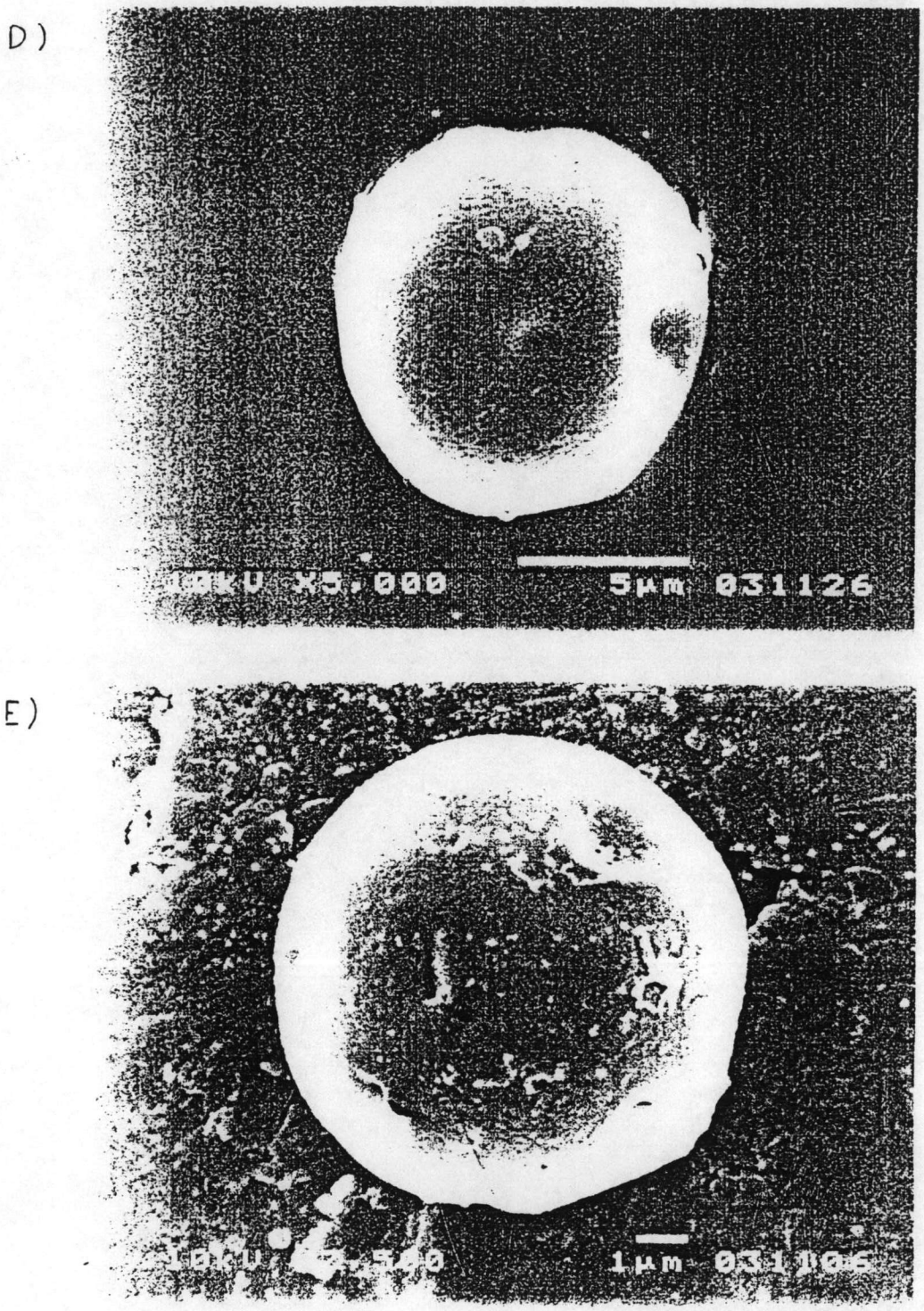


Figure 46 SEM micrographs of unloaded microparticles degeadation prepared with PLGA 75:25 incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

A)

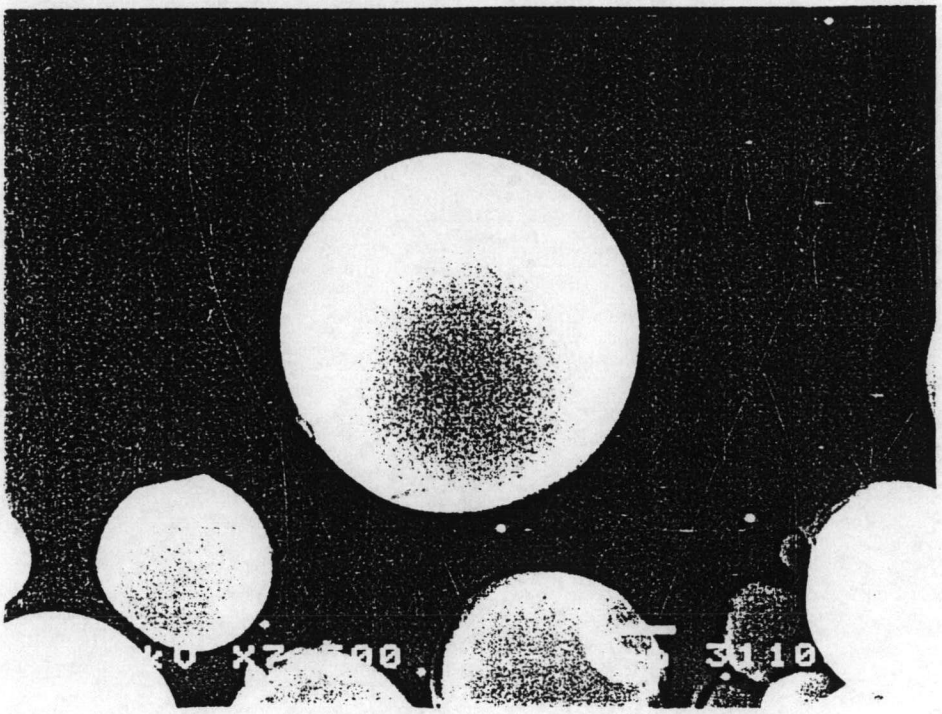


Figure 47 SEM micrographs of unloaded microparticles degeadation prepared with PLGA 50:50 incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

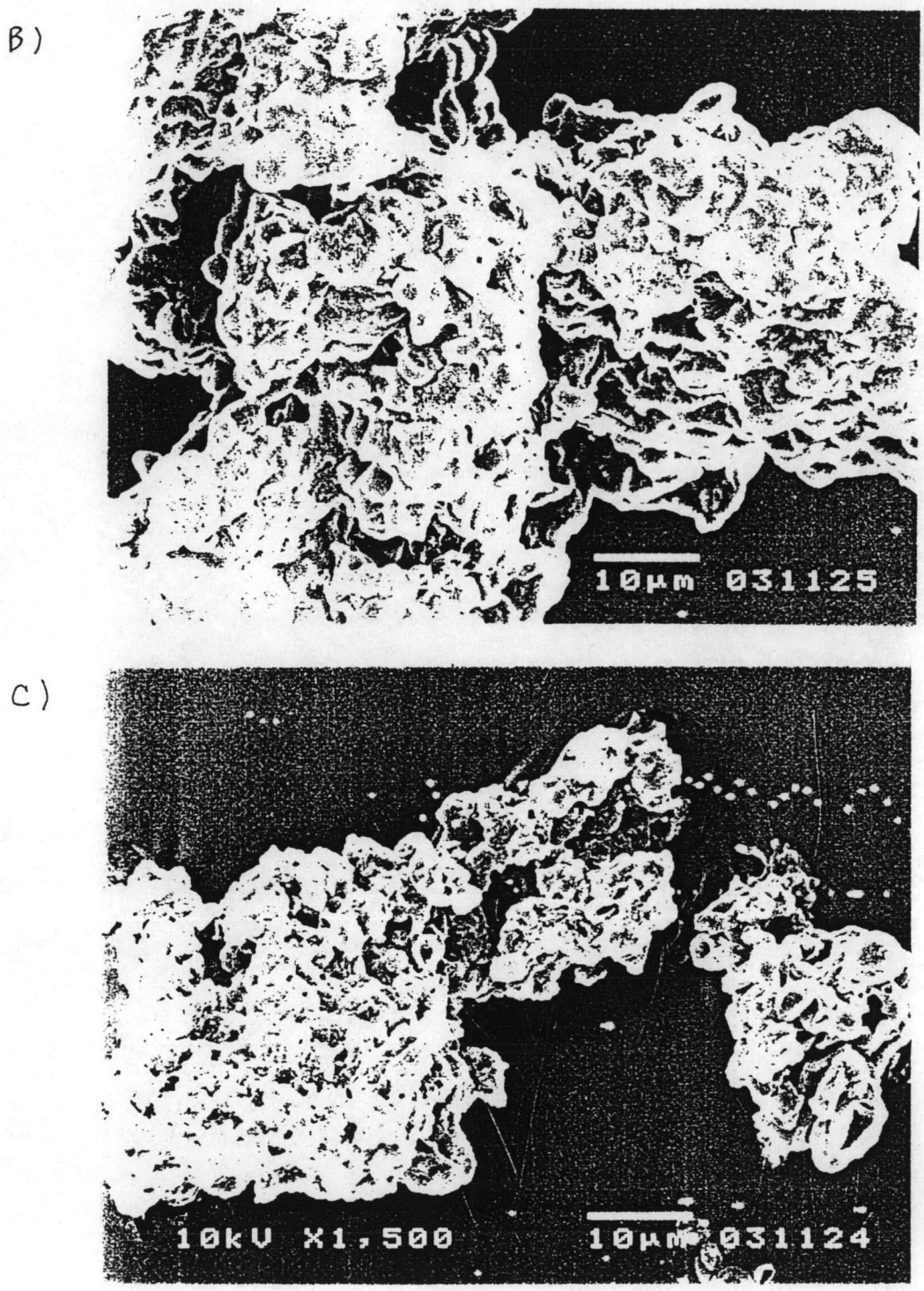


Figure 47 SEM micrographs of unloaded microparticles degeadation prepared with PLGA 50:50 incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

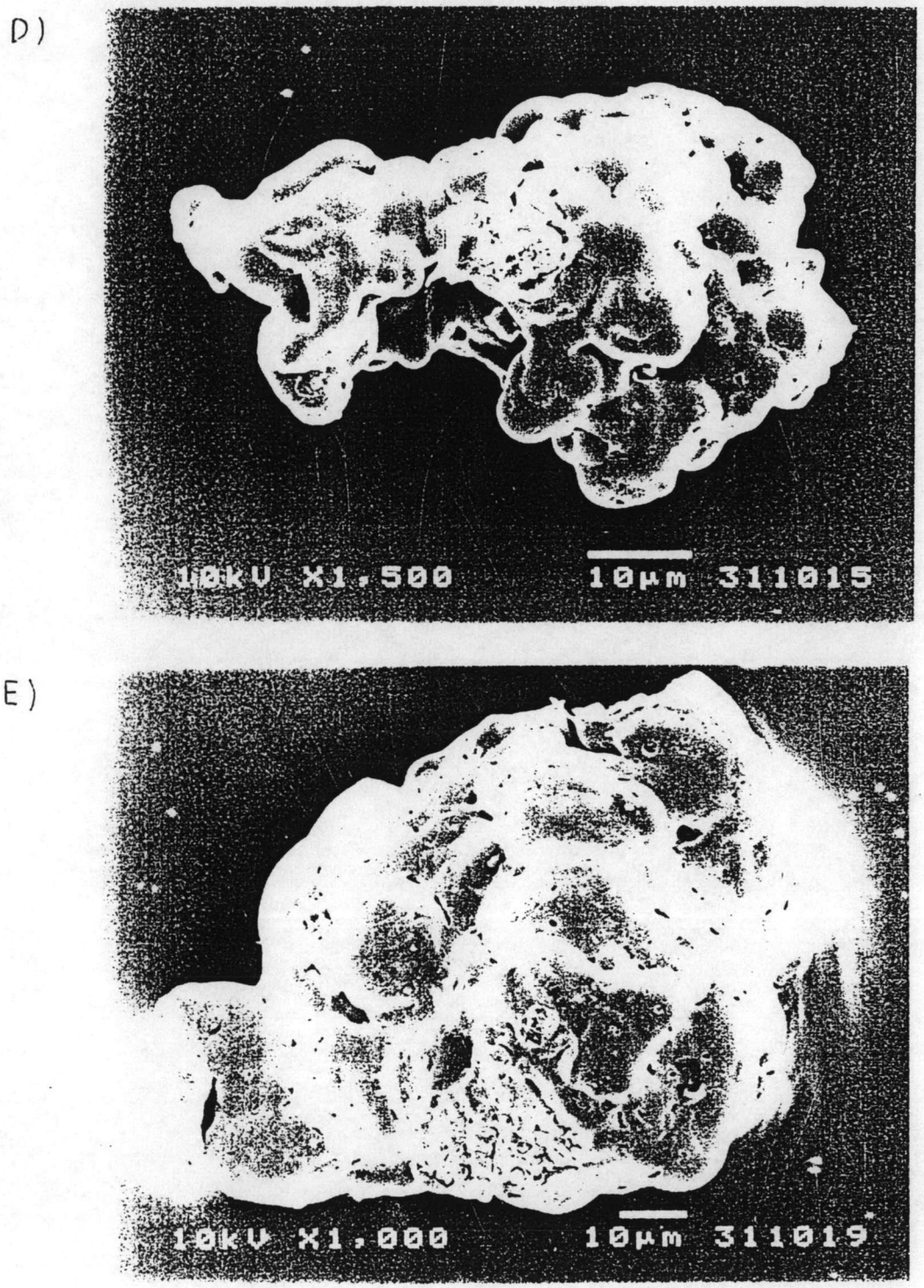


Figure 47 SEM micrographs of unloaded microparticles degeadation prepared with PLGA 50:50 incubated in PBS pH 7.4 at different time periods : A) week 0 , B) week 4 , C) week 8 , D) week 12 , E) week 16

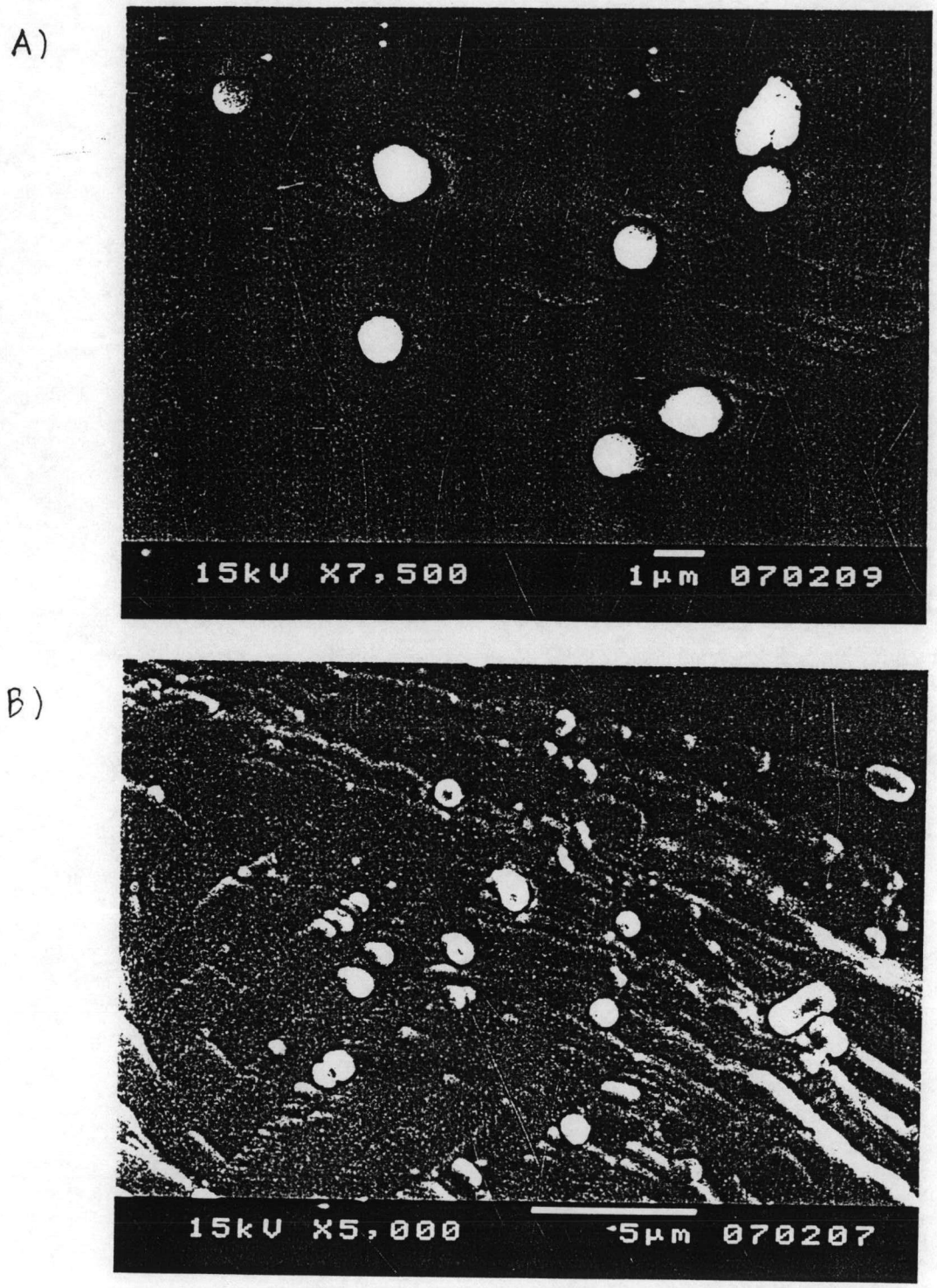


Figure 48 SEM micrographs of unloaded and loaded CS microparticles : A) unloaded microparticles , B) loaded microparticles of LMW CS , C) loaded microparticles of MMW CS , D) loaded microparticles of HMW CS

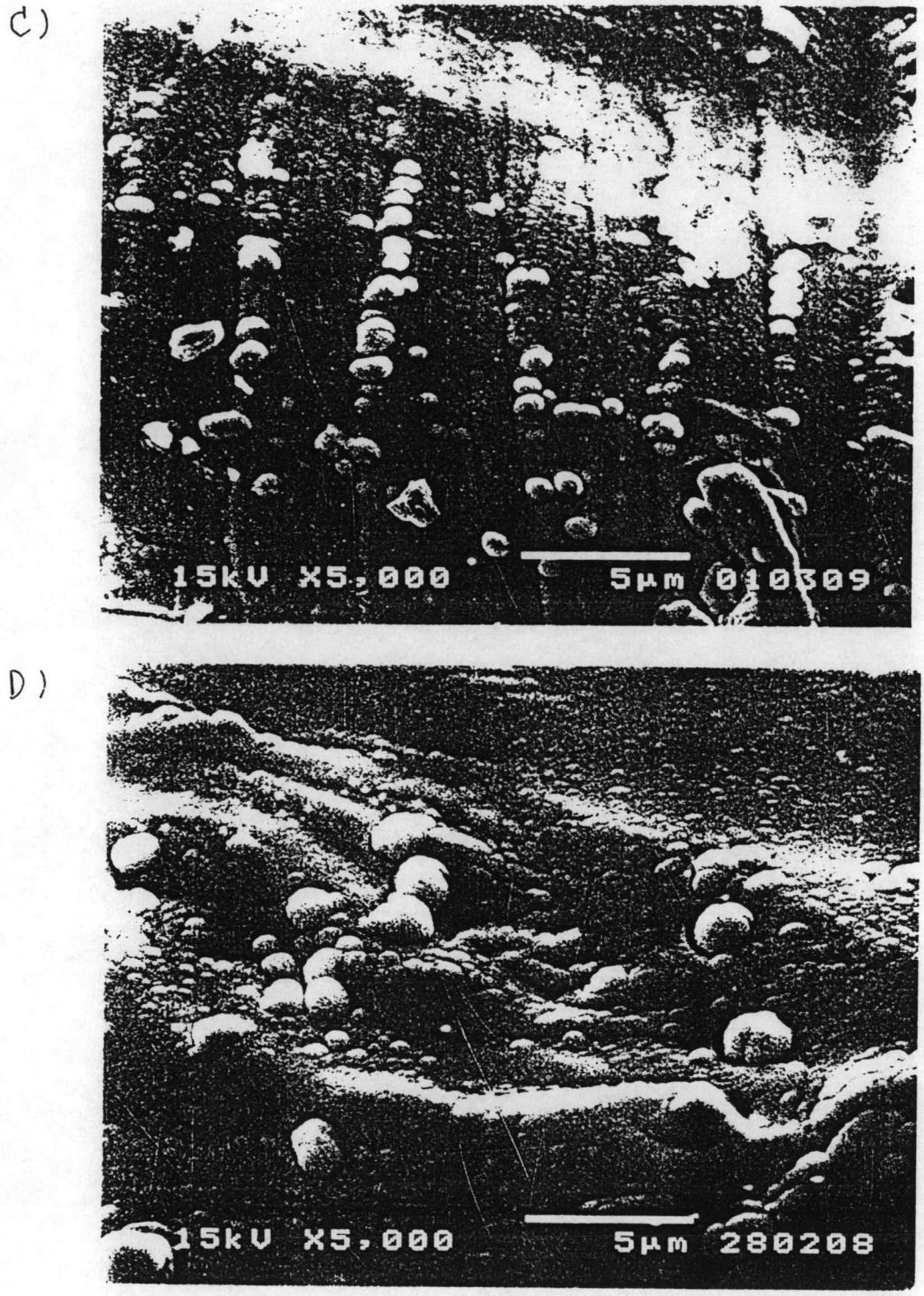


Figure 48 SEM micrographs of unloaded and loaded CS microparticles : A) unloaded microparticles , B) loaded microparticles of LMW CS , C) loaded microparticles of MMW CS , D) loaded microparticles of HMW CS

Table 27 Particle size of antigen-loaded chitosan microparticles

Polymer	Concentration (%)	Ratio aqueous : oil phase	Antigen added (ug)	Particle size (um)			Uniformity
				D(v,0.1)	D(v,0.5)	D(v,0.9)	
LMW CS	1	1 : 5	250	1.36	6.73	31.14	1.359E+00
			500	1.41	6.72	41.94	1.738E+00
			750	1.50	7.74	51.24	1.824E+00
		2 : 5	250	3.90	8.83	22.39	6.321E-01
			500	1.86	8.94	67.03	2.988E+00
			750	1.91	9.10	65.12	3.022E+00
	2	1 : 5	250	1.36	9.25	41.15	1.304E+00
			500	4.83	9.64	16.70	3.830E-01
			750	1.77	10.19	32.91	9.423E-01
	3	1 : 5	250	1.59	11.25	62.27	1.537E+00
			500	2.91	11.57	23.99	5.544E-01
			750	1.66	11.63	42.35	2.233E+00
MMW CS	1	1 : 5	250	1.44	8.01	47.67	1.595E+00
			500	1.44	8.14	50.78	1.661E+00
			750	1.59	9.04	81.00	5.205E+00
	2	1 : 5	250	3.91	10.81	19.35	4.358E-01
			500	4.21	11.03	20.30	4.585E-01
			750	4.28	11.26	22.88	1.392E+00
HMW CS	1	1 : 5	250	1.80	10.61	34.20	9.455E-01
			500	3.88	10.82	19.48	4.390E-01
			750	4.17	10.97	20.02	4.508E-01

distribution was varied in each formulation. The size of microparticles was dependent upon several factors such as molecular weight, polymer concentration, aqueous : oil phase ratio and amount of antigen added in formulations. The increase in amount of antigen added in preparations slightly increased the particle size. Furthermore, the increment in aqueous-to-oil phase ratio, polymer concentration and polymer molecular weight extremely increase the particle size.

1.2.3 Antigen content and entrapment efficiency

The antigen content and percent entrapment efficiency from chitosan microparticles are summarized in Table 28. The entrapment efficiency from all batches was more than 50%. However, there were several factors affecting the antigen content and entrapment efficiency. These included amount of antigen added in preparations, aqueous : oil phase ratio, polymer concentration and polymer molecular weight. The antigen content profoundly increased by increasing the amount of antigen added in formulations. On the other hand, antigen content decreased by increasing aqueous : oil phase ratio, polymer concentration and polymer molecular weight. However, the relationship of amount of antigen added, aqueous : oil phase ratio, polymer concentration and polymer molecular weight could not clearly conclude.

1.2.4 In vitro antigen release

In vitro release profiles of antigen from chitosan microparticles are depicted in figures 49-51. The antigen release was dependent upon molecular weight and concentration of chitosan. The antigen release from HMW CS microparticles was slower than that from LMW CS microparticles. In addition, the increment in polymer concentration reduced antigen release. The effect of aqueous : oil phase ratio on antigen release was observed. The decrease of antigen release was seen when increase in aqueous : oil phase ratio.

Table 28 Antigen content and entrapment efficiency from chitosan microparticles

Polymer	Concentration (%)	Ratio aqueous:oil phase	Antigen added (ug)	Antigen loading (SD) (ug)	Entrapment efficiency (%)	Antigen content (ug /100 mg sample)
LMW CS	1	1 : 5	250	191.03 (13.19)	76.41	106.36
			500	348.52 (22.85)	69.70	226.17
			750	499.59 (30.55)	66.61	318.21
		2 : 5	250	182.61 (12.86)	73.04	49.41
			500	345.38 (25.14)	69.08	98.57
			750	499.44 (33.92)	66.59	141.85
	2	1 : 5	250	189.02 (13.38)	75.61	53.19
			500	348.65 (25.87)	69.73	101.50
			750	504.75 (35.14)	67.30	144.21
	3	1 : 5	250	185.80 (13.81)	74.32	33.29
			500	342.68 (24.55)	68.54	63.0.
			750	508.66 (36.04)	67.82	91.70
MMW CS	1	1 : 5	250	172.05 (12.07)	68.82	99.68
			500	328.26 (22.60)	65.65	198.34
			750	474.91 (31.94)	63.32	293.70
		2 : 5	250	163.24 (10.92)	65.30	45.37
			500	321.74 (21.73)	64.35	86.61
			750	465.86 (34.16)	62.11	129.37
	2	1 : 5	250	166.21 (10.24)	66.48	45.36
			500	329.29 (22.19)	65.86	90.96
			750	472.58 (31.18)	63.01	132.52
		2 : 5	250	156.96 (10.95)	62.78	30.27
			500	315.72 (21.50)	63.14	58.4?
			750	468.35 (32.88)	62.45	92.35
HMW CS	1	1 : 5	250	116.10 (8.66)	46.44	73.11
			500	243.22 (17.74)	48.64	157.43
			750	398.92 (28.20)	53.19	241.92

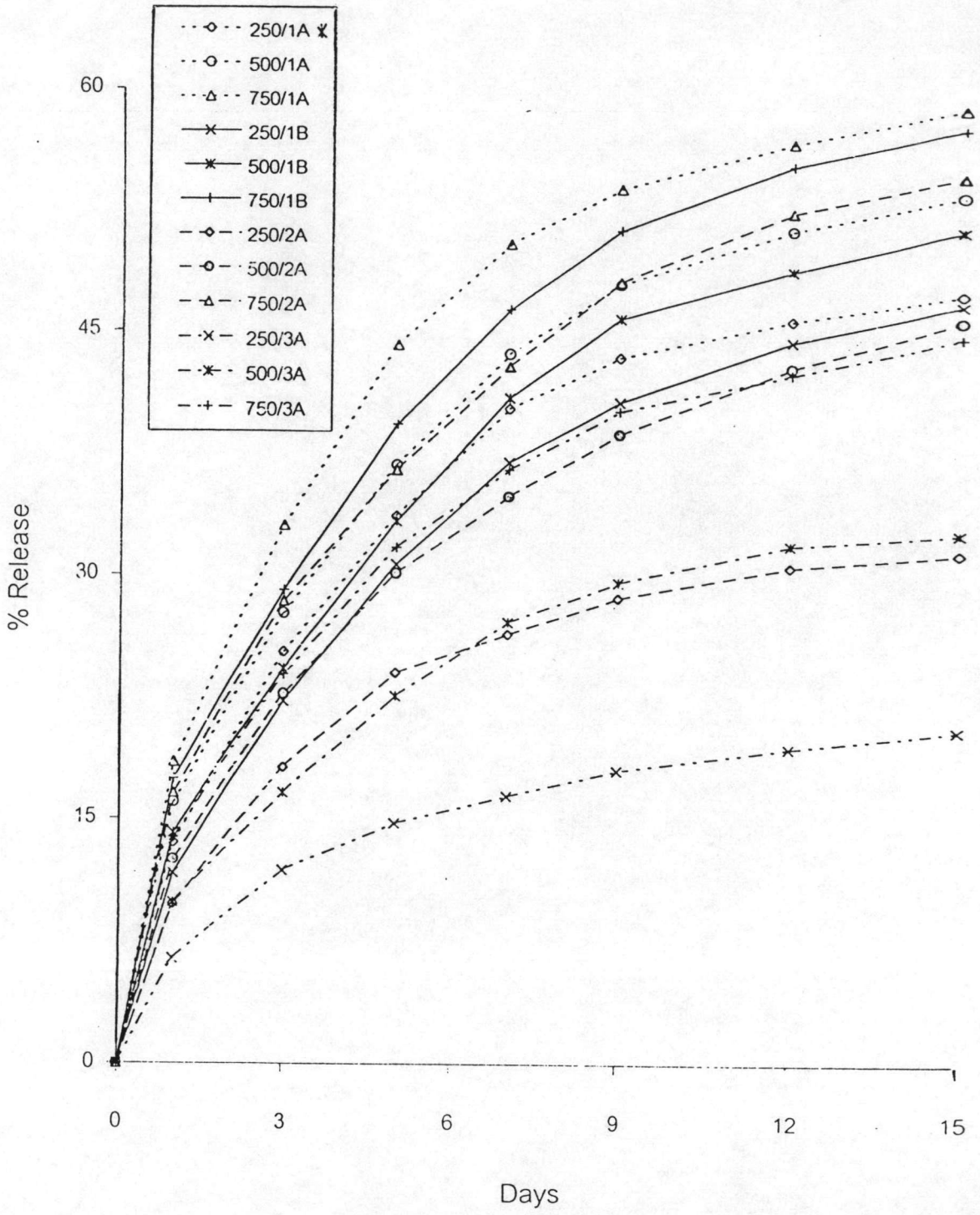


Figure 49 In vitro antigen release profiles of microparticles prepared with LMW chitosan polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

1, 2, 3 = chitosan concentration (%)

A, B = aqueous : oil phase ratio ; A = 1:5, B = 2:5

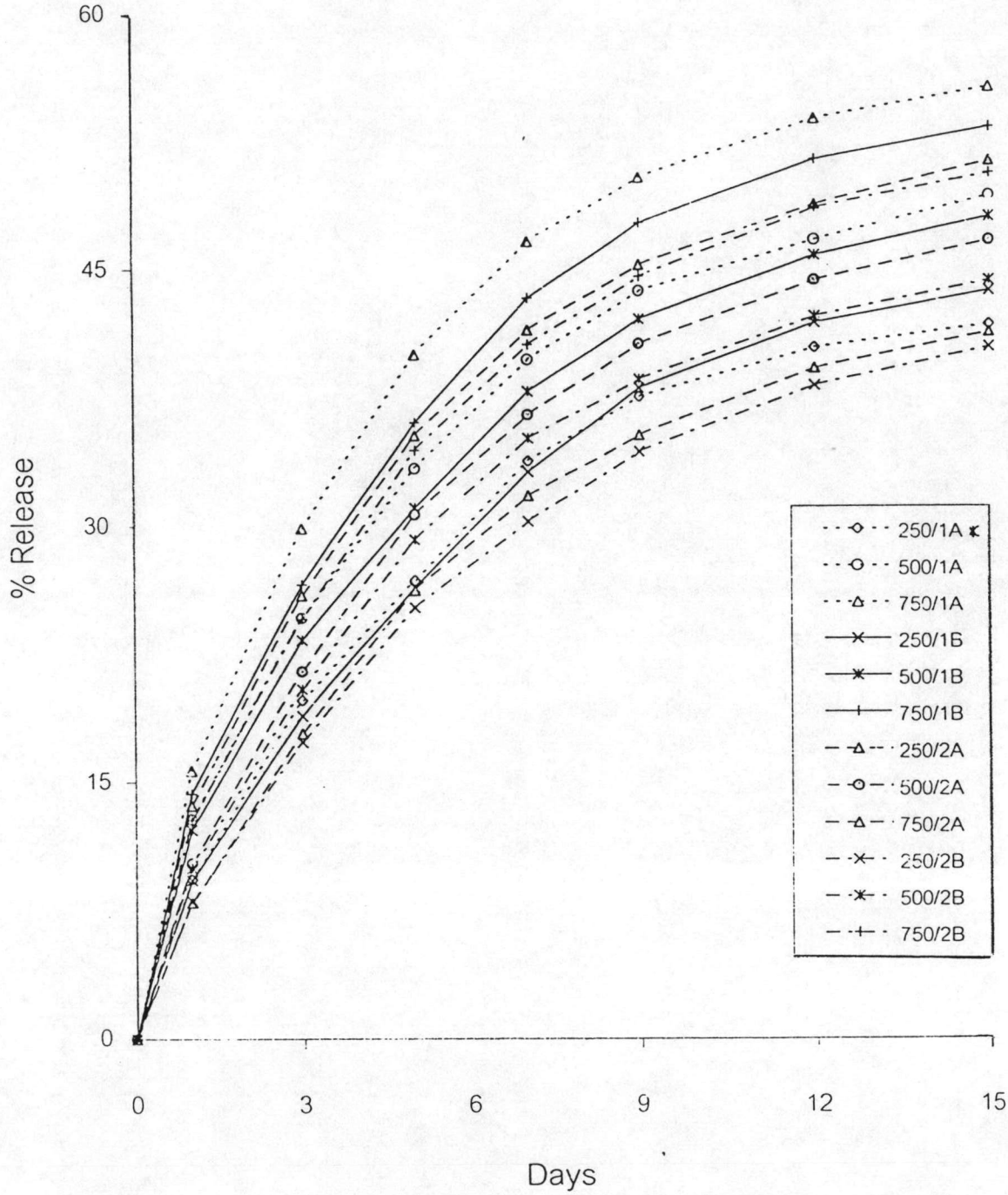


Figure 50 In vitro antigen release profiles of microparticles prepared with MMW chitosan polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

1, 2 = chitosan concentration (%)

A, B = aqueous : oil phase ratio ; A = 1:5, B = 2:5

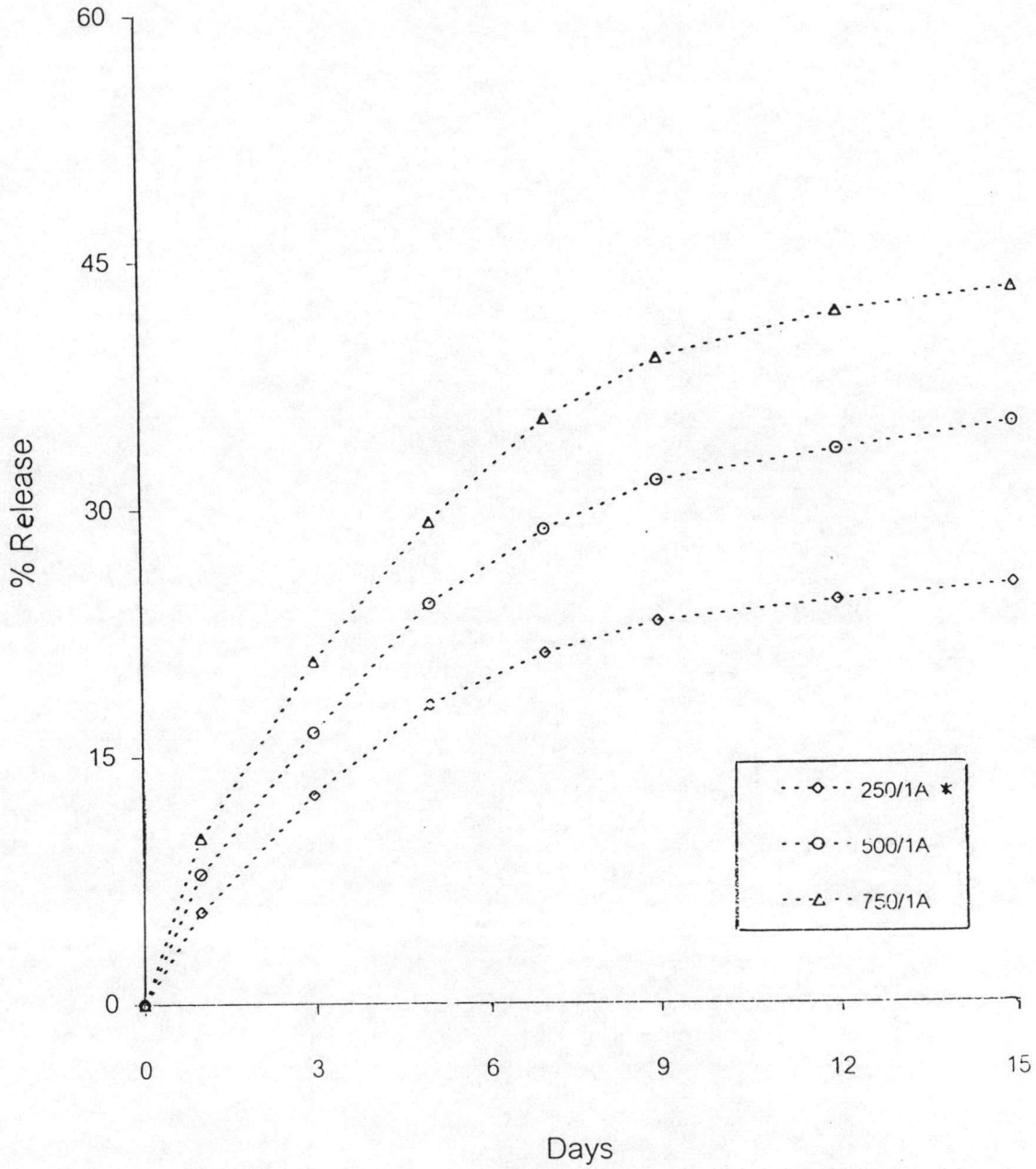


Figure 51 In vitro antigen release profiles of microparticles prepared with HMW chitosan polymer in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

1 = chitosan concentration (%)

A = aqueous : oil phase ratio ; A = 1:5

1.2.5 Antigen integrity

The SDS-PAGE from JE antigen encapsulated in chitosan microparticles is illustrated in figure 52. It expressed three bands of protein with molecular weight approximately of 30, 35 and 50 kDa. These protein bands resembled the bands from pure JE antigen. No change in pattern of JE antigen was appeared. There was no additional band to indicate the presence of molecular weight aggregates or fragments. Therefore, the structural integrity of JE antigen was not apparently affected by encapsulation process.

1.2.6 Polymer degradation

In vitro degradation study of unloaded chitosan microparticles is expressed in Table 29 and figure 53. The microparticles produced from three different molecular weight of chitosan were studied. It could be seen that molecular weight tremendously decreased within the first month after incubation in PBS pH7.4 medium. Then the degradation was gradually decreased with time until the fourth month. In addition, the molecular weight loss in each polymer was of different rate. The microparticles produced from HMW CS had the highest loss in molecular weight followed by those from MMW and LMW CS with approximately of 75 % weight loss in four weeks.

2. Stability assessment

2.1 Polyester microparticles

The SEM micrographs of microparticles kept at 40°C for 1 month are shown in figure 54. These expressed that high temperature had a slight effect on shape and surface morphology of microparticles. The shape and surface morphology of DL-PLA and PLGA 85:15 microparticles were still spherical with smooth surface whereas PLGA 75:25 and PLGA 50:50 microparticles still had spherical shape but the surface

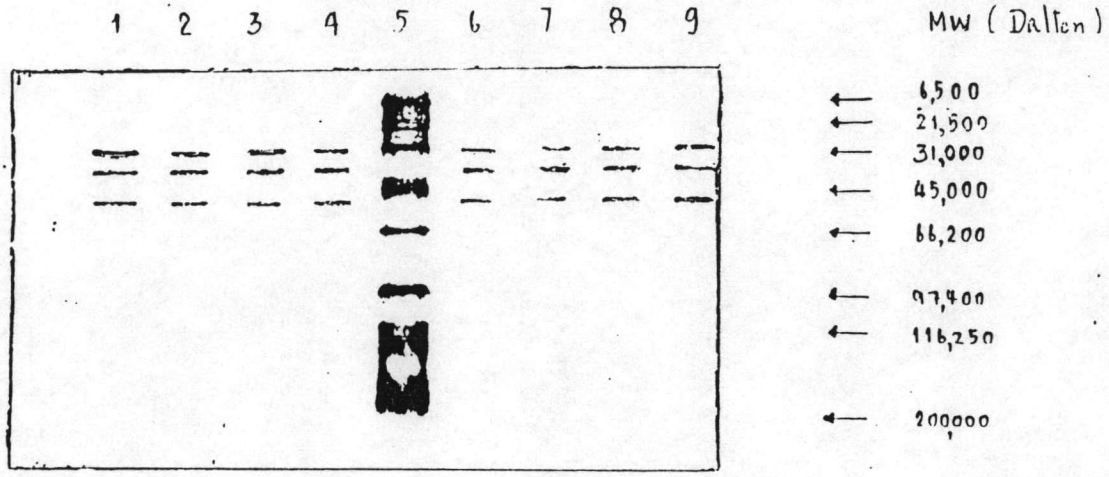


Figure 52 Structural integrity of JE antigen encapsulated in chitosan microparticles ; JE antigen from LMW CS : lane 1,7, MMW CS : lane 2,8, HMW CS : lane 3,9, pure JE antigen : lane 4,6 and protein standard marker : lane 5

Table 29 Molecular weight of chitosan polymers at different time of degradation

Time (weeks)	Results	Polymer		
		LMW CS	MMW CS	HMW CS
0	MW (Dalton)	41025	56718	83261
	Polydispersity	4.779	6.300	7.617
4	MW (Dalton)	10483	12203	16775
	Polydispersity	2.785	2.957	3.046
8	MW (Dalton)	9711	10502	12778
	Polydispersity	2.617	2.859	2.656
12	MW (Dalton)	9307	9924	10940
	Polydispersity	2.492	2.444	2.545
16	MW (Dalton)	9138	9625	9855
	Polydispersity	2.611	2.636	2.493

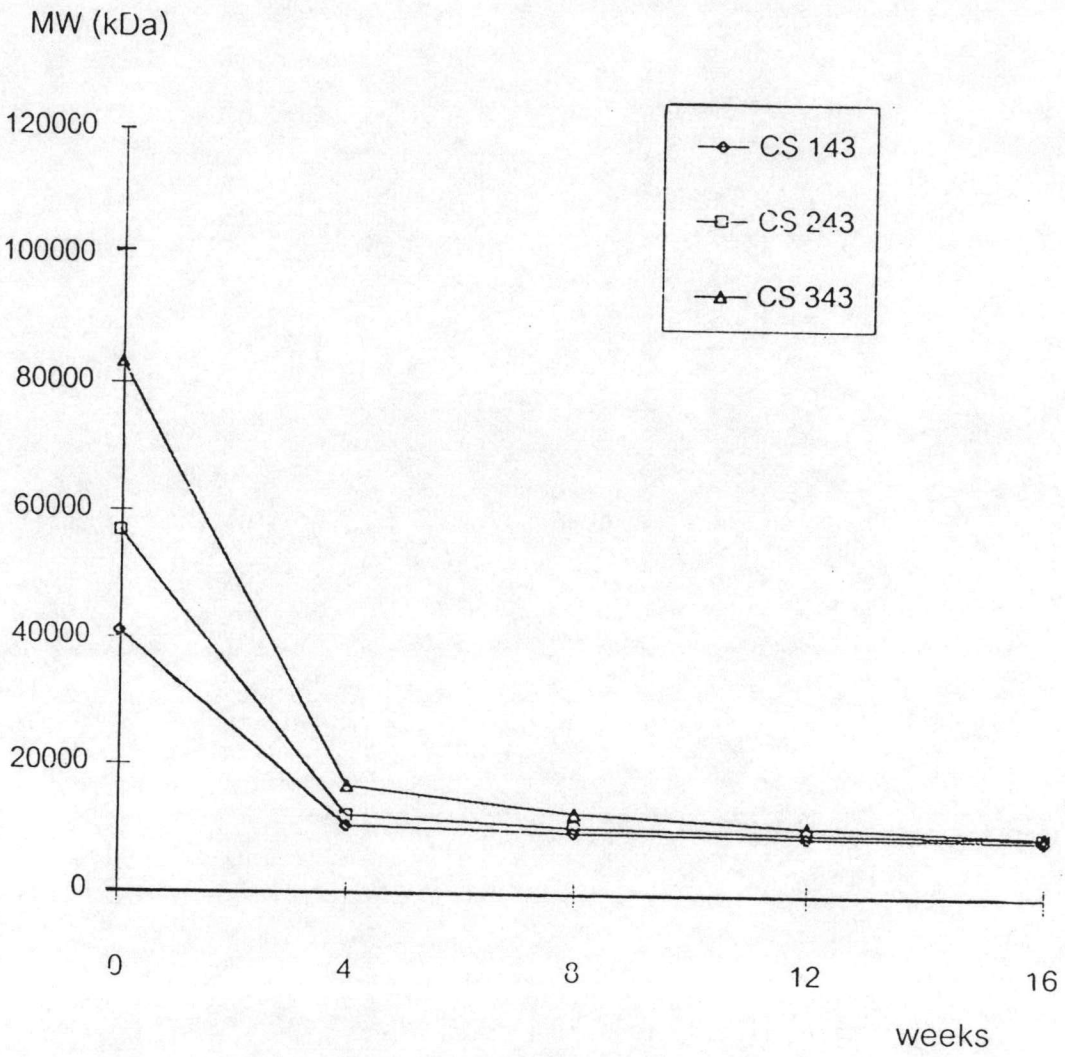


Figure 53 In vitro degradation profiles of unloaded chitosan microparticles incubated in PBS pH 7.4 at different time intervals

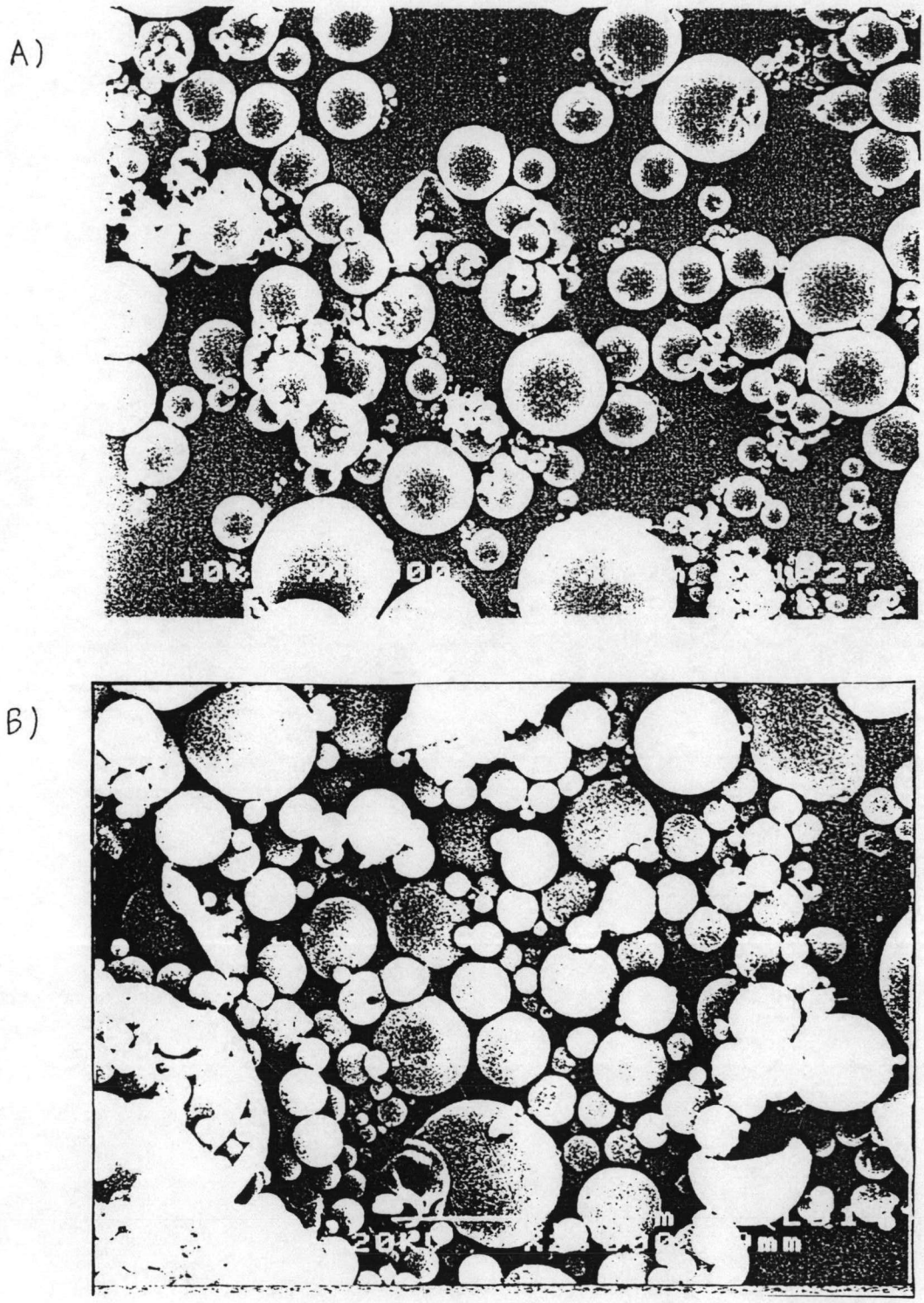
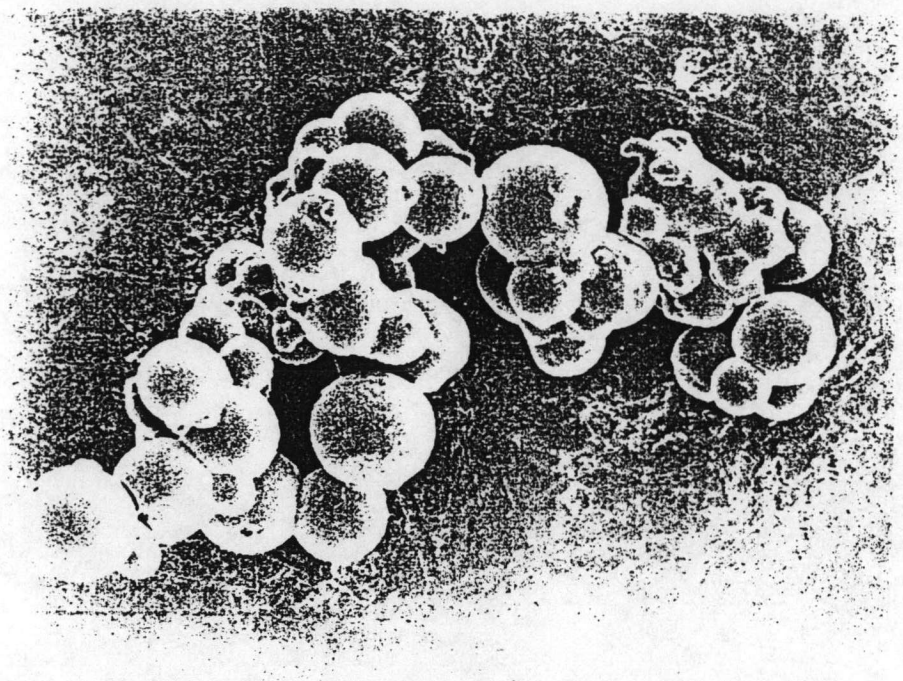


Figure 54 SEM micrographs of PLGA microparticles kept at 40°C for 1 month : A) DL-PLA microparticles , B) PLGA 85:15 microparticles , C) PLGA 75:25 microparticles , D) PLGA 50:50 microparticles

C)



D)

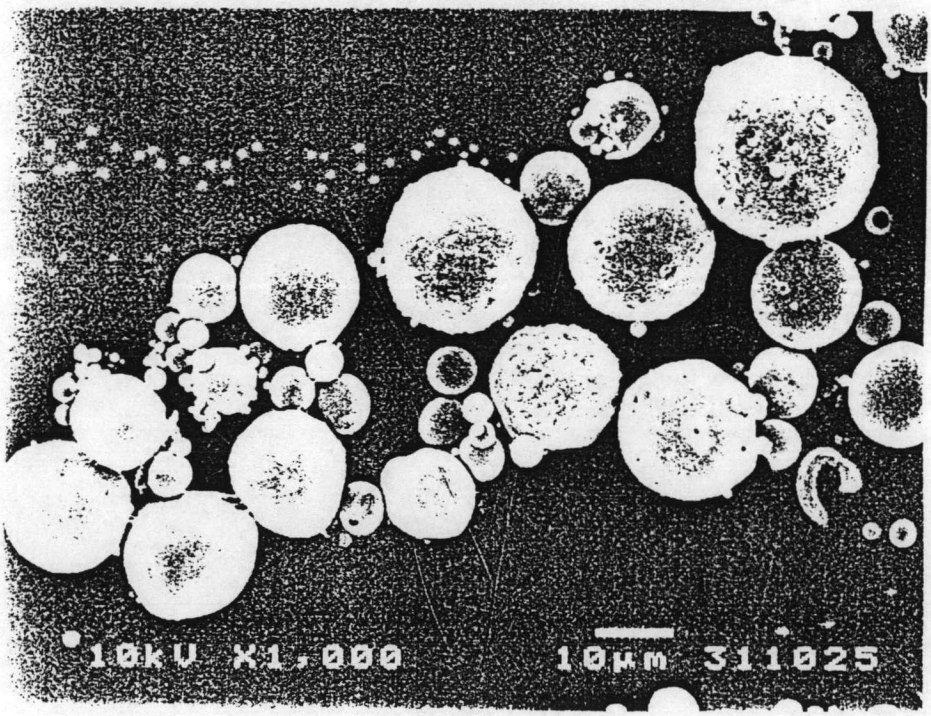


Figure 54 SEM micrographs of PLGA microparticles kept at 40°C for 1 month : A) DL-PLA microparticles , B) PLGA 85:15 microparticles , C) PLGA 75:25 microparticles , D) PLGA 50:50 microparticles

morphology was rather rough and uneven. In addition, the aggregation of microparticles was observed. This was supported by the data from particle size analysis. The size of microparticles after storage at high temperature is expressed in Table 30. The average particle size was larger and size distribution was wider than the freshly prepared microparticles.

The antigen content and in vitro release of antigen from microparticles slightly decreased after stability study. The antigen content is summarized in Tables 31-34. In vitro release profiles are depicted in figures 55-66. The data indicated that the freeze-dried microparticles still maintained their antigen content after storage of 40°C for 1 month. Further, result from SDS-PAGE revealed the structural integrity of antigen. No additional bands were observed as shown in figure 67. No aggregation and no smaller molecular weight fragments of protein were appeared. Therefore, high temperature did not influence on structural integrity of JE antigen.

2.2 Chitosan microparticles

The shape and surface morphology of chitosan microparticles determined by Cryo-SEM were slightly altered as shown in figure 68. It seemed that there was no effect of high temperature on shape and surface morphology of microparticles. However, freeze-fractured SEM micrographs showed some degree of aggregation of microparticles. The particle size characterized after stability study is denoted in Table 35. The size increased in comparison to freshly prepared microparticles. The particle size distribution was of great variation in each preparation. Some preparations exhibited narrow particle size distribution while some exhibited wide distribution.

The antigen content of microparticles is denoted in Table 36. The result from in vitro release profiles shown in figures 69-71 expressed that the release of

Table 30 Particle size of antigen-loaded PLGA microparticles kept at 40°C for 1 month

Polymer	Concentration (%)	Sonicate (output)	Antigen added (ug)	Particle size (um)			Uniformity
				D(v,0.1)	D(v,0.5)	D(v,0.9)	
DL-PLA	1.5	15	250	2.88	11.45	24.33	5.682E-01
			500	3.48	11.69	25.27	7.433E-01
			750	4.07	11.78	24.49	6.876E-01
	3	15	750	3.39	14.31	38.25	1.057E+00
	5	15	750	2.93	15.57	66.28	1.813E+00
PLGA 85:15	1.5	15	250	5.72	11.07	19.10	3.754E-01
			500	4.14	11.14	20.22	9.294E-01
			750	3.02	11.27	22.43	5.215E-01
	3	15	750	5.20	13.98	29.49	7.125E-01
	5	15	750	4.23	15.17	32.37	7.944E-01
PLGA 75:25	1.5	15	250	3.14	10.59	21.52	5.503E-01
			500	3.96	10.86	19.35	4.321E-01
			750	4.03	10.98	19.63	4.365E-01
	3	15	750	3.84	12.39	24.92	6.305E-01
	5	15	750	5.26	14.01	29.61	7.103E-01
PLGA 50:50	1.5	15	250	1.72	8.70	30.10	1.693E+00
			500	3.90	8.84	22.43	6.325E-01
			750	2.59	9.17	20.60	5.985E-01
	3	15	750	1.83	10.99	35.95	9.538E-01
	5	15	750	4.11	12.32	24.35	5.078E-01

Table 31 Antigen content from DL- PLA microparticles kept at 40°C for 1 month

Polymer	Concentration (%)	Antigen added (ug)	Sonicate output	Antigen content (SD) (ug /100 mg sample)
DL-PLA	1.5	250	9	63.31 (2.71)
			12	66.93 (2.33)
			15	69.44 (3.15)
		500	9	168.30 (7.06)
			12	165.41 (6.10)
			15	170.75 (7.75)
		750	9	305.65 (12.10)
			12	300.44 (10.20)
			15	305.40 (12.40)
	3	250	9	35.56 (2.61)
			12	37.94 (1.97)
			15	37.11 (2.25)
		500	9	87.87 (3.12)
			12	89.42 (2.24)
			15	86.04 (3.45)
		750	9	163.17 (7.41)
			12	165.74 (6.85)
			15	162.76 (7.78)
	5	250	9	24.19 (1.06)
			12	25.42 (1.22)
			15	25.75 (1.25)
		500	9	54.30 (2.34)
			12	54.85 (2.25)
			15	55.96 (1.98)
		750	9	97.95 (5.10)
			12	98.64 (4.59)
			15	101.02 (6.22)

Table 32 Antigen content from PLGA 85:15 microparticles kept at 40°C for 1 month

Polymer	Concentration (%)	Antigen added (ug)	Sonicate output	Antigen content (SD) (ug /100 mg sample)
PLGA 85:15	1.5	250	9	72.66 (4.33)
			12	70.56 (3.50)
			15	76.23 (3.13)
		500	9	163.94 (9.21)
			12	166.76 (7.66)
			15	170.42 (7.00)
		750	9	310.73 (14.10)
			12	314.07 (12.80)
			15	321.08 (10.95)
	3	250	9	41.35 (2.02)
			12	42.33 (1.78)
			15	45.16 (1.65)
		500	9	98.04 (4.60)
			12	99.44 (3.75)
			15	104.48 (5.69)
		750	9	174.59 (9.25)
			12	177.53 (7.43)
			15	178.36 (8.02)
	5	250	9	26.77 (1.17)
			12	28.26 (0.95)
			15	26.91 (0.81)
		500	9	58.79 (2.95)
			12	58.56 (3.25)
			15	60.13 (3.01)
		750	9	101.42 (7.22)
			12	103.30 (6.73)
			15	105.23 (5.30)

Table 33 Antigen content from PLGA 75:25 microparticles kept at 40°C for 1 month

Polymer	Concentration (%)	Antigen added (ug)	Sonicate output	Antigen content (SD) (ug /100 mg sample)
PLGA 75:25	1.5	250	9	82.62 (3.60)
			12	83.68 (3.38)
			15	84.55 (3.65)
		500	9	192.56 (8.45)
			12	194.10 (11.00)
			15	199.24 (10.20)
		750	9	367.78 (15.41)
			12	349.60 (15.65)
			15	361.74 (13.96)
	3	250	9	42.50 (2.05)
			12	44.42 (1.45)
			15	44.78 (1.88)
		500	9	103.40 (4.73)
			12	104.96 (5.20)
			15	100.52 (4.95)
		750	9	171.65 (10.10)
			12	184.51 (8.85)
			15	188.76 (9.21)
	5	250	9	27.12 (1.04)
			12	28.63 (0.95)
			15	29.07 (1.12)
		500	9	63.19 (3.44)
			12	60.29 (3.75)
			15	64.58 (2.87)
		750	9	105.28 (7.85)
			12	109.36 (6.90)
			15	105.55 (6.95)

Table 34 Antigen content from PLGA 50:50 microparticles kept at 40°C for 1 month

Polymer	Concentration (%)	Antigen added (ug)	Sonicate output	Antigen content (SD) (ug /100 mg sample)
PLGA 50:50	1.5	250	9	83.49 (5.23)
			12	89.78 (4.98)
			15	90.72 (4.22)
		500	9	215.21 (12.10)
			12	209.17 (10.13)
			15	223.10 (11.70)
		750	9	352.33 (17.85)
			12	358.71 (18.10)
			15	359.29 (15.65)
	3	250	9	51.58 (2.28)
			12	54.15 (2.51)
			15	51.55 (2.10)
		500	9	111.65 (6.40)
			12	117.41 (5.56)
			15	116.02 (6.10)
		750	9	194.96 (11.60)
			12	202.62 (10.95)
			15	205.74 (12.11)
	5	250	9	31.37 (1.19)
			12	29.39 (1.65)
			15	32.00 (1.30)
		500	9	70.14 (4.10)
			12	73.58 (3.67)
			15	72.01 (3.70)
		750	9	114.52 (6.25)
			12	111.94 (5.21)
			15	117.99 (5.79)

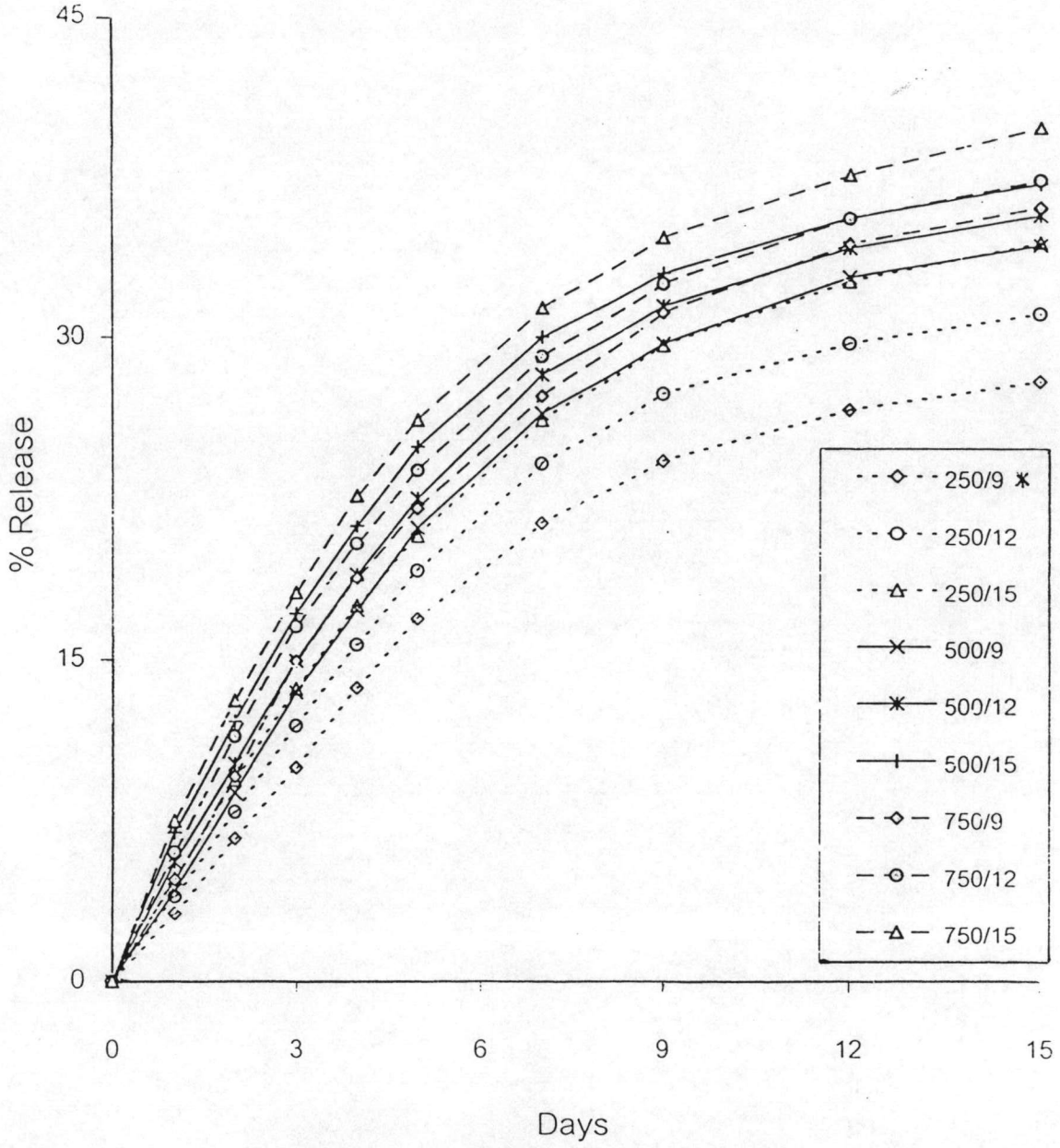


Figure 55 In vitro antigen release profiles of microparticles prepared with 1.5 % DL - PLA polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

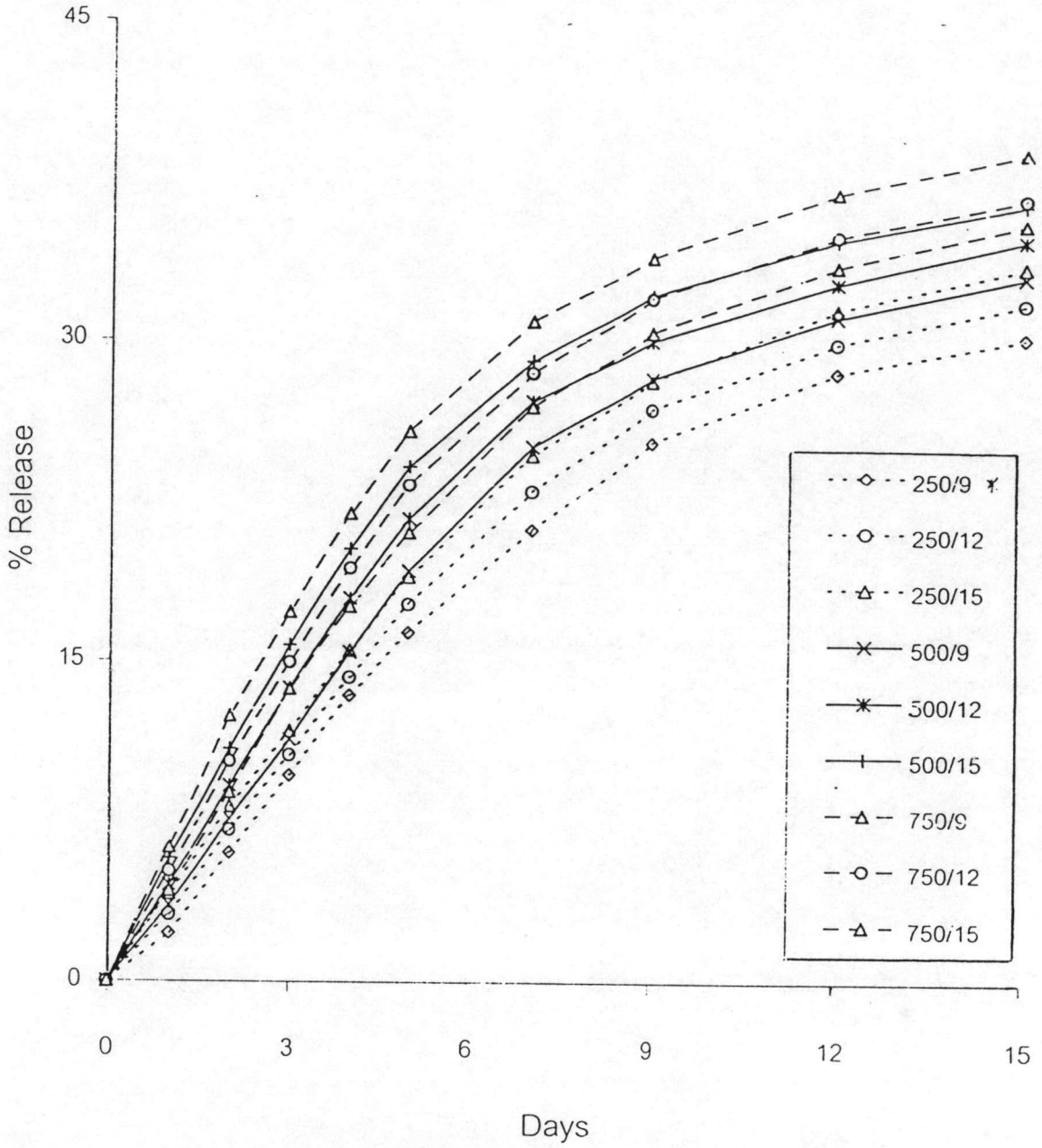


Figure 56 In vitro antigen release profiles of microparticles prepared with 3 % DL - PLA polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

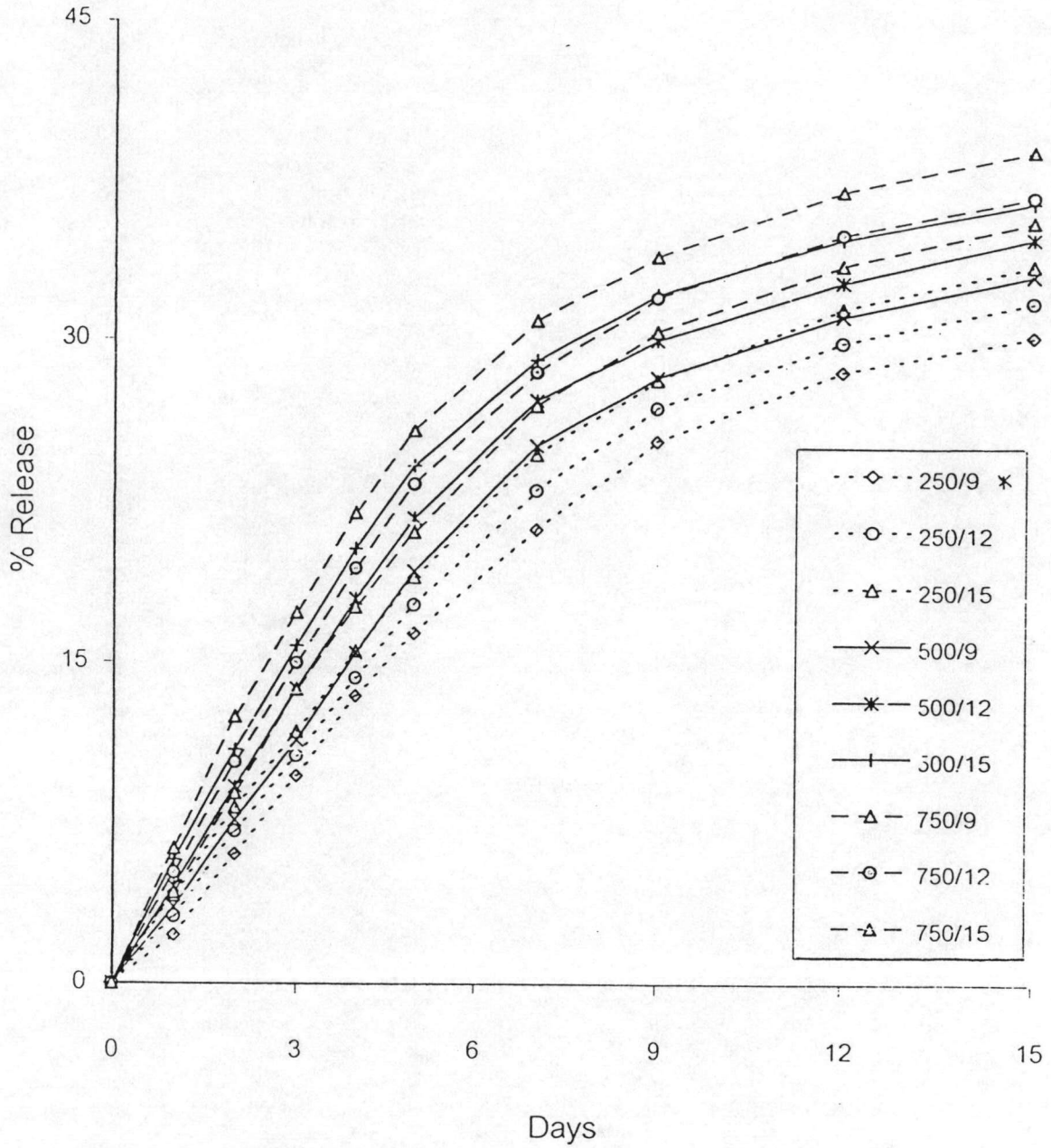


Figure 57 In vitro antigen release profiles of microparticles prepared with 5 % DL - PLA polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

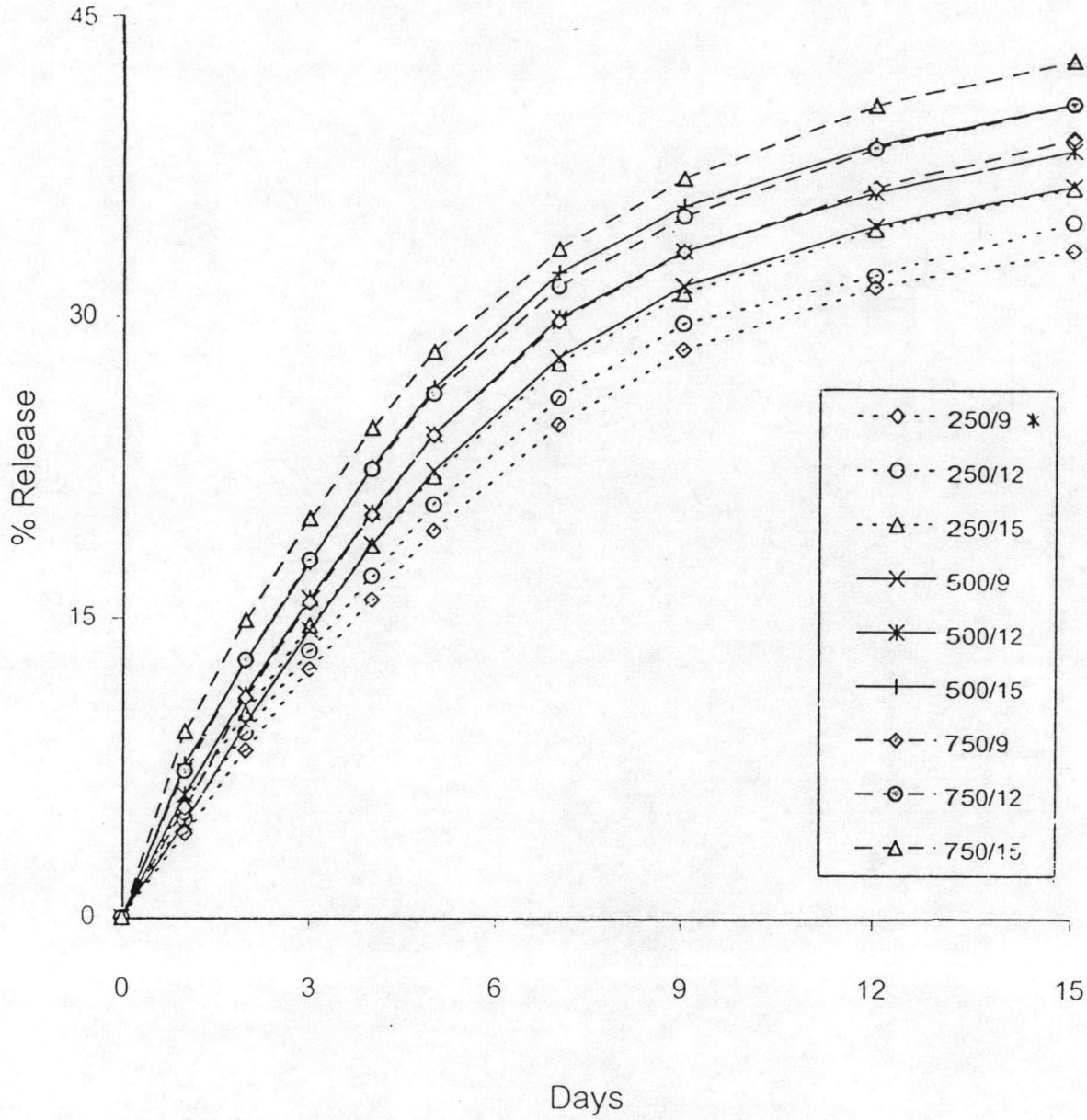


Figure 58 In vitro antigen release profiles of microparticles prepared with 1.5 % PLGA 85:15 polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

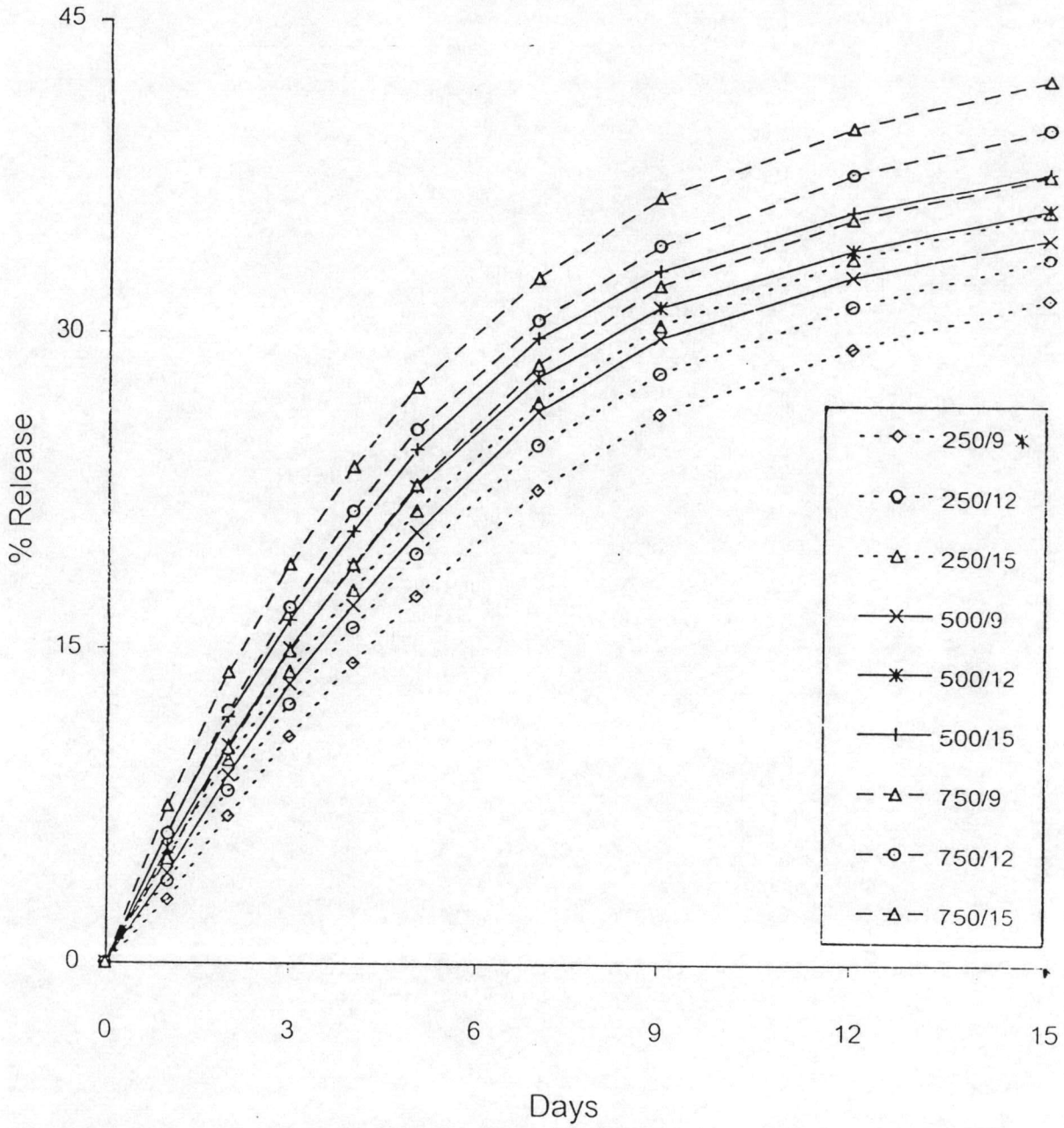


Figure 59 In vitro antigen release profiles of microparticles prepared with 3 % PLGA 85:15 polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

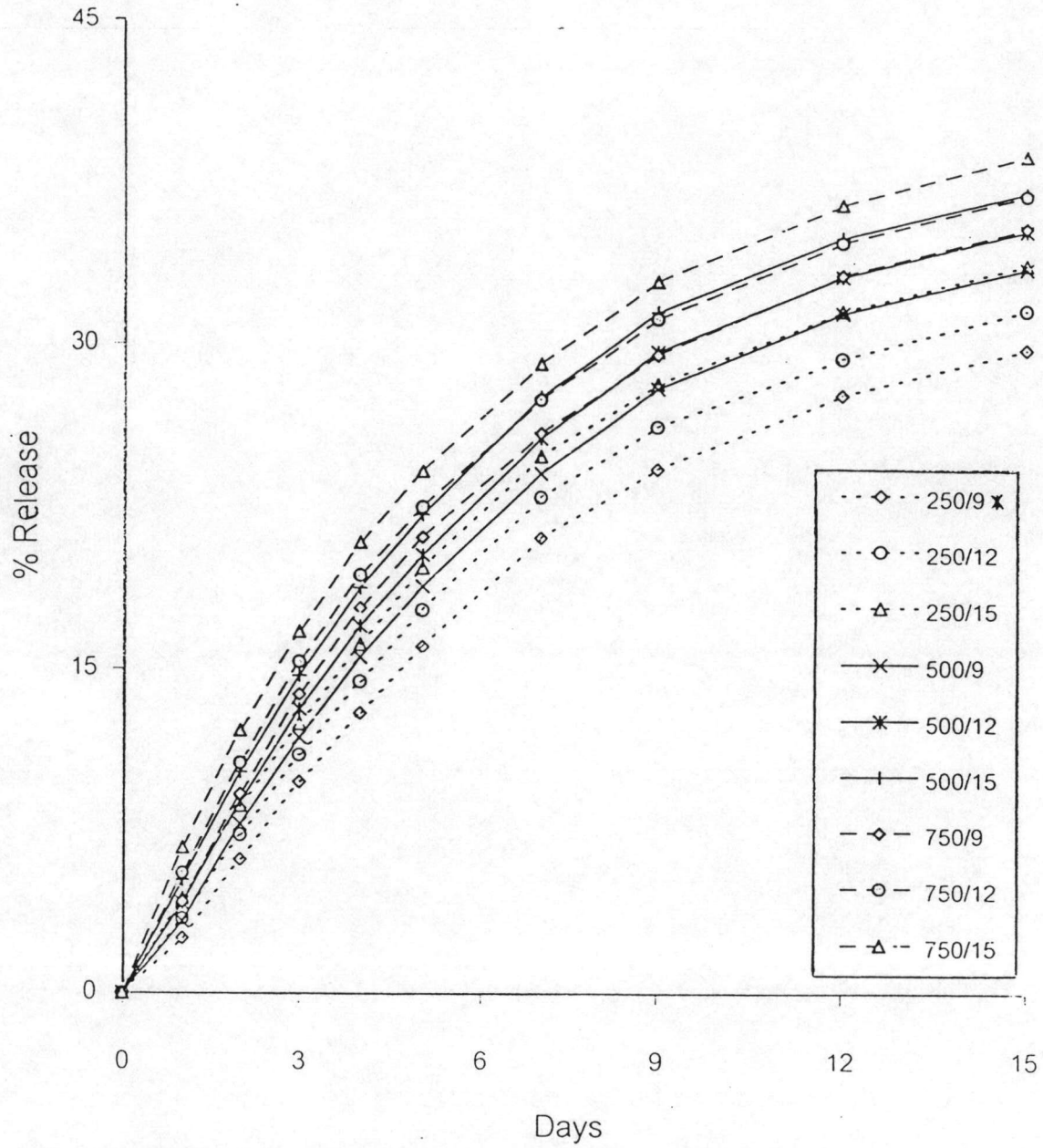


Figure 60 In vitro antigen release profiles of microparticles prepared with 5 % PLGA 85:15 polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

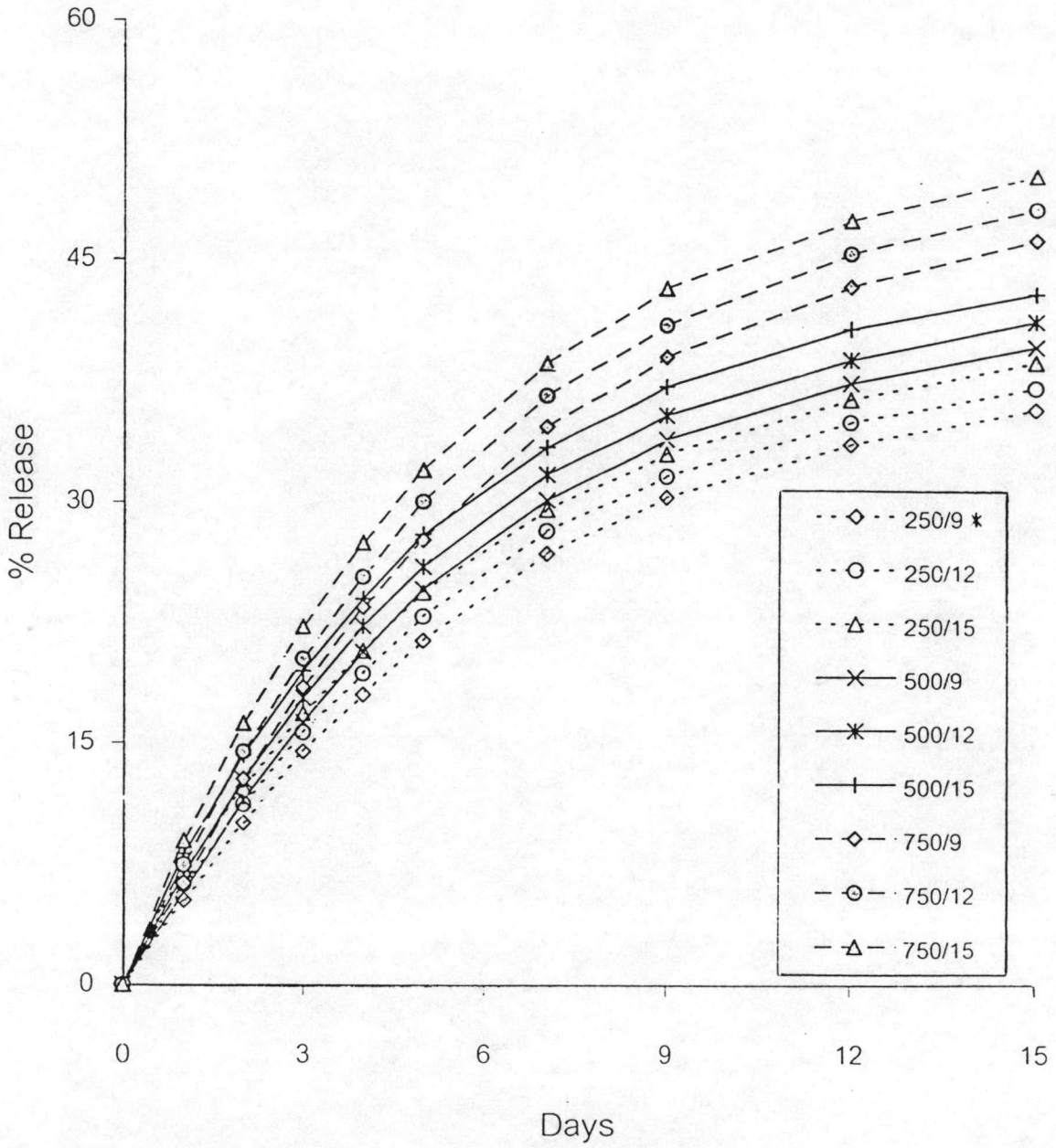


Figure 61 In vitro antigen release profiles of microparticles prepared with 1.5 % PLGA 75:25 polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

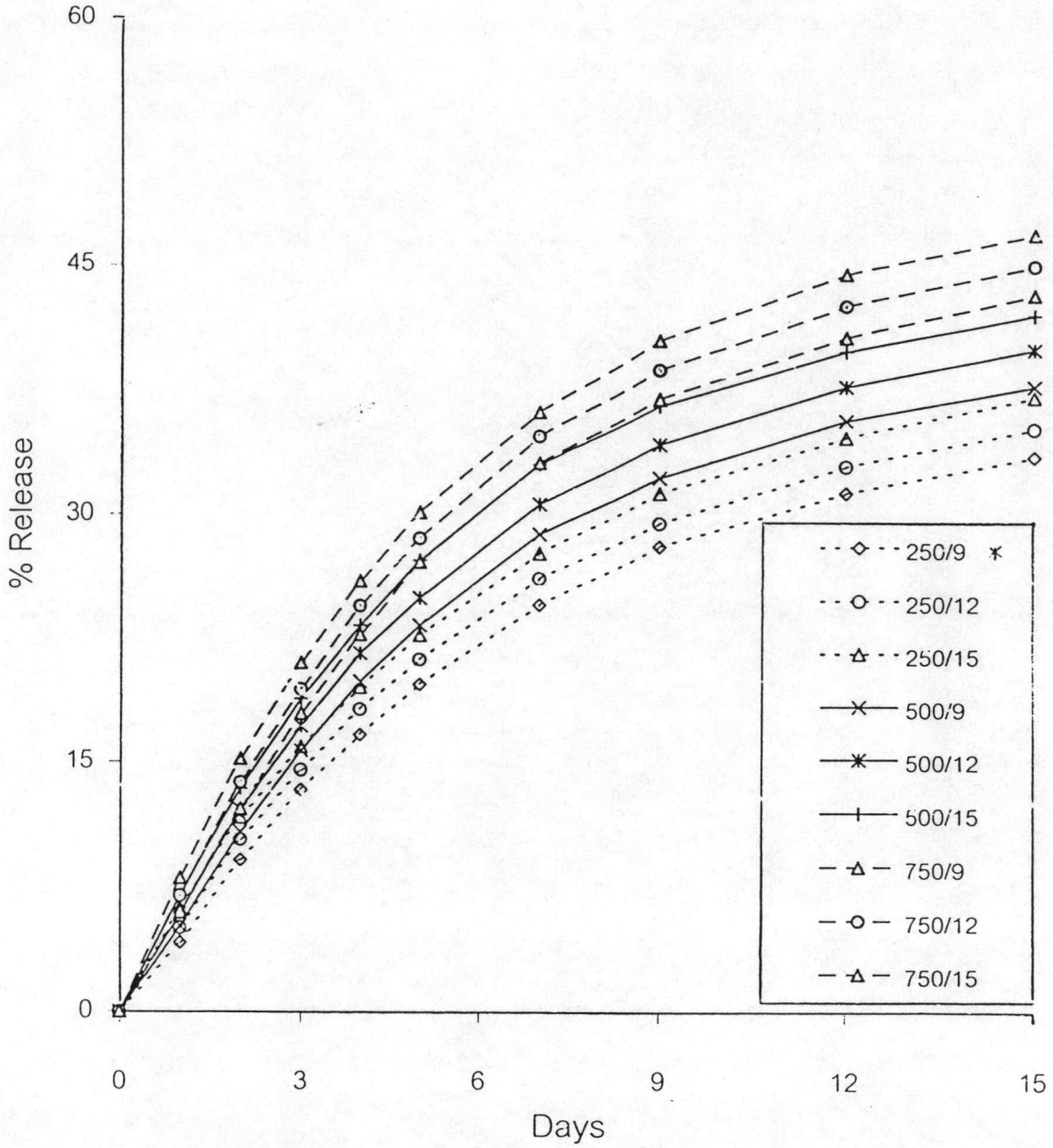


Figure 62 In vitro antigen release profiles of microparticles prepared with 3 % PLGA 75:25 polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

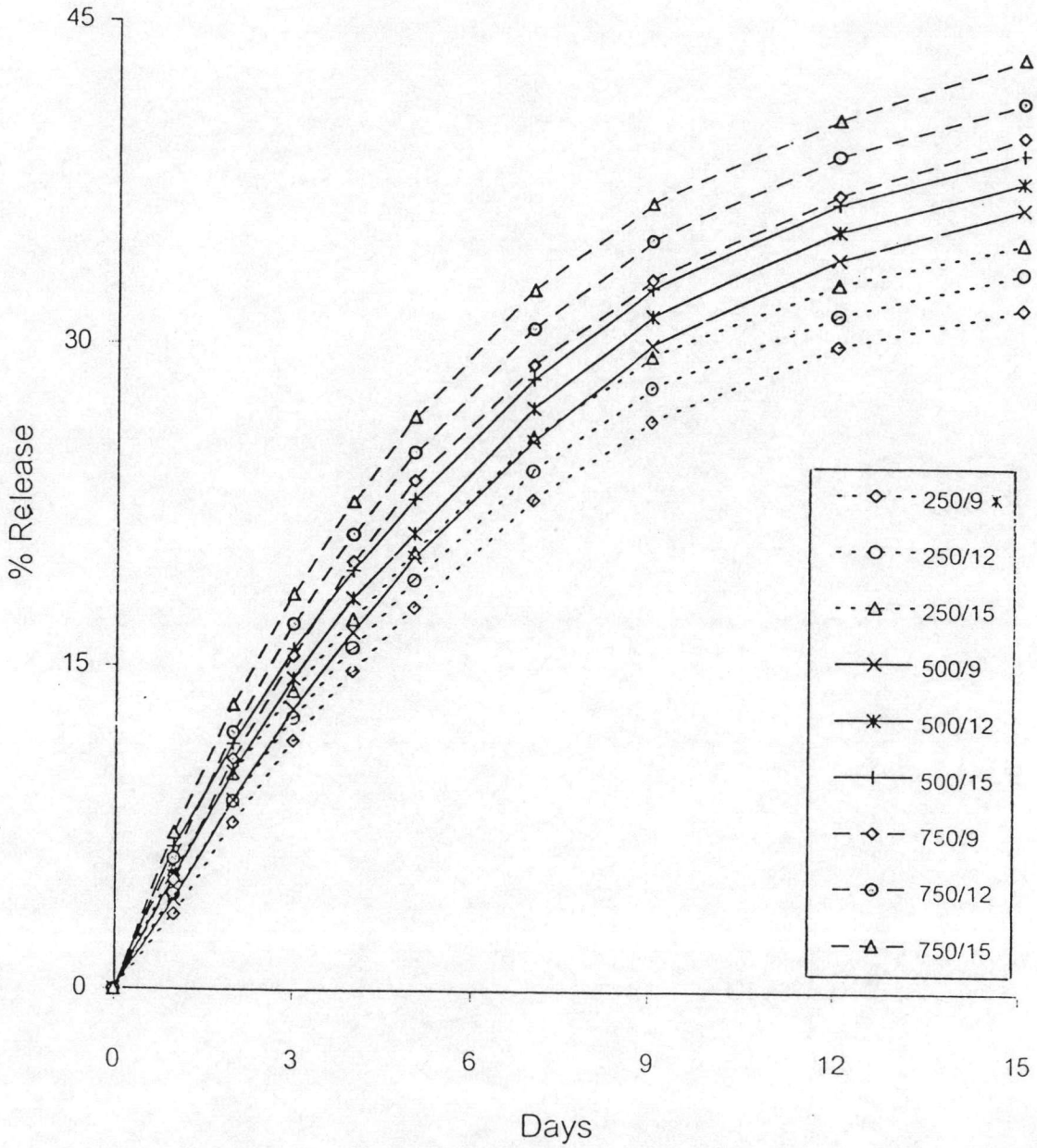


Figure 63 In vitro antigen release profiles of microparticles prepared with 5 % PLGA 75:25 polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

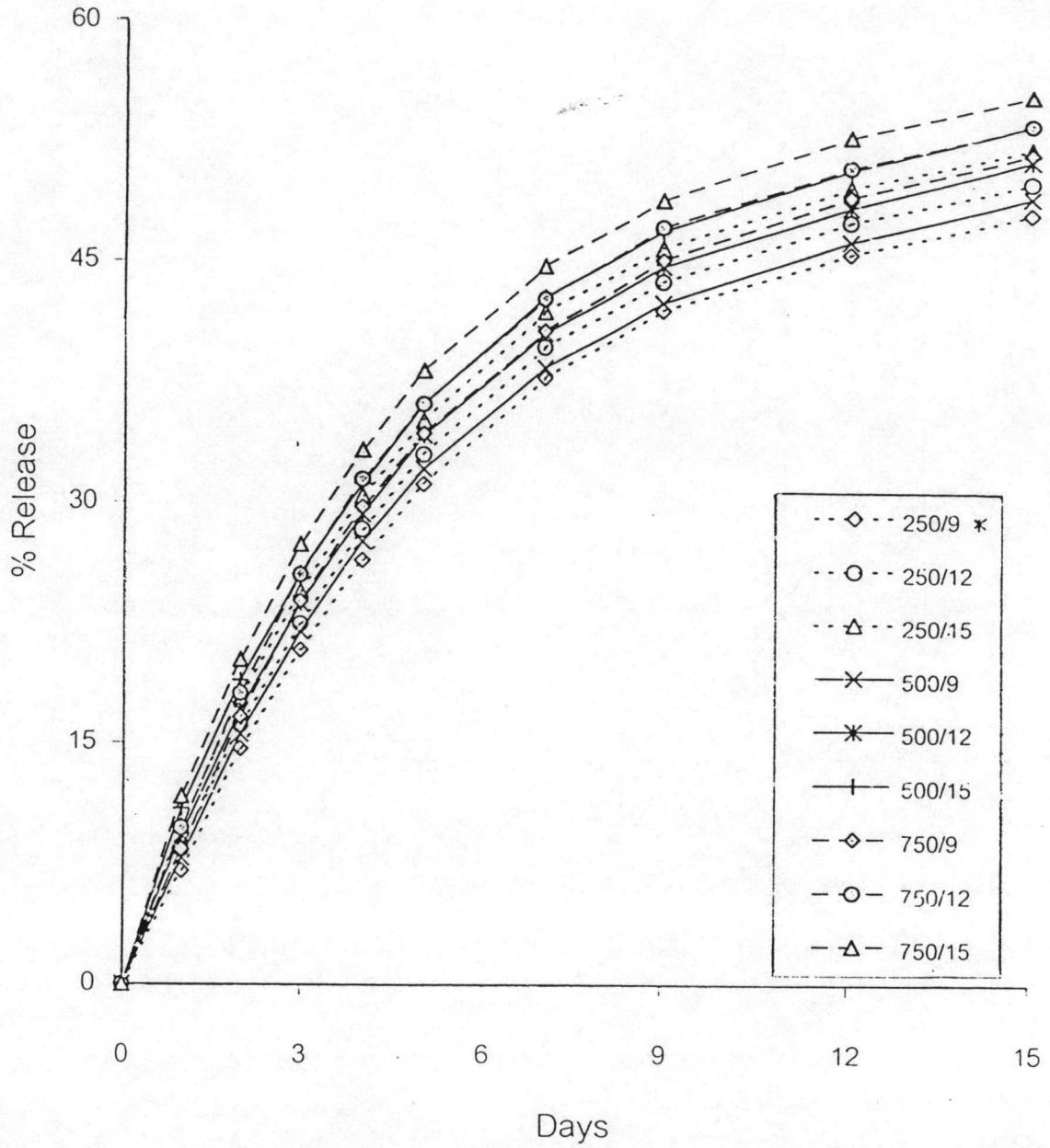


Figure 64 In vitro antigen release profiles of microparticles prepared with 1.5 % PLGA 50:50 polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

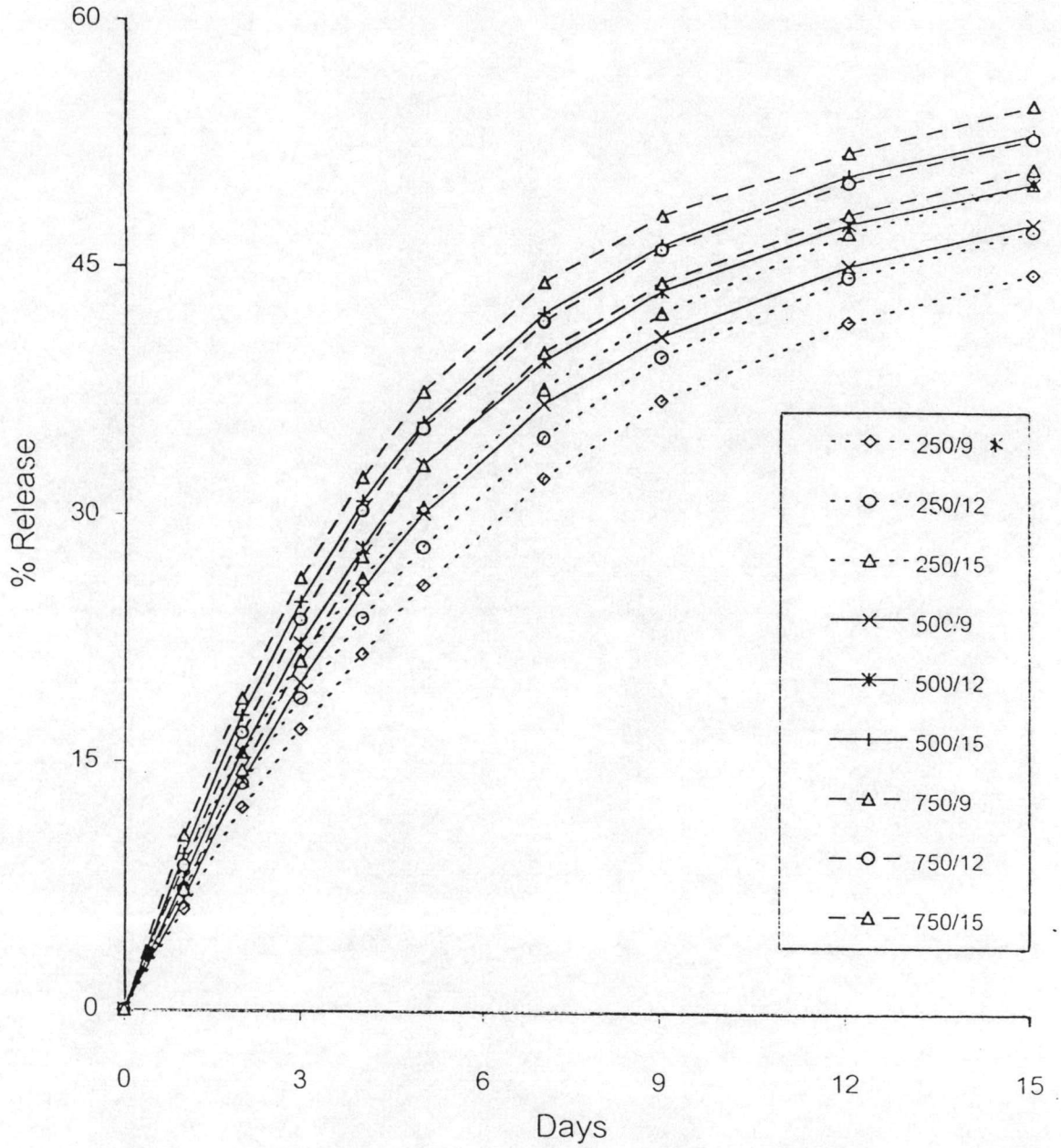


Figure 65 In vitro antigen release profiles of microparticles prepared with 3 % PLGA 50:50 polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

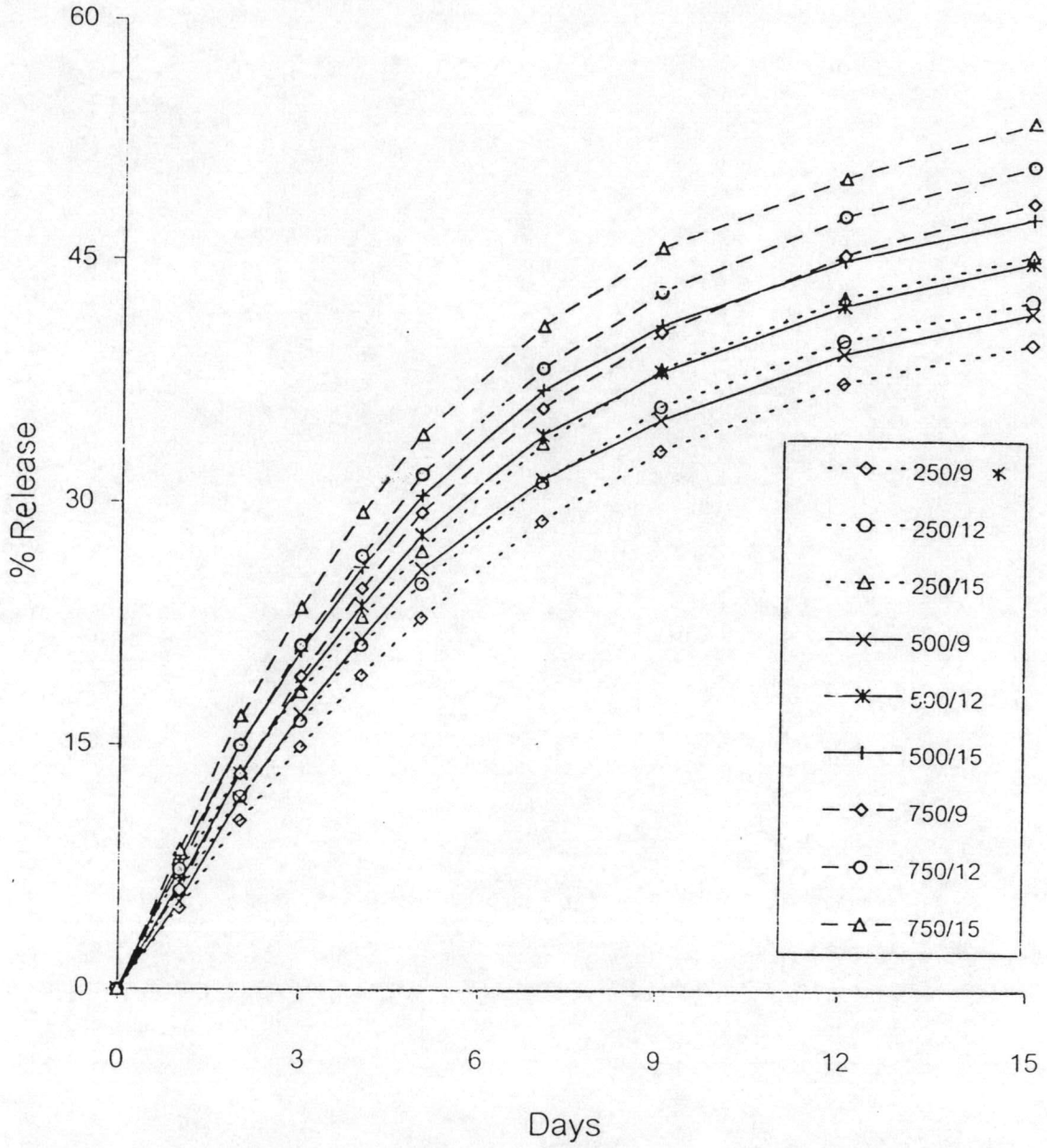


Figure 66 In vitro antigen release profiles of microparticles prepared with 5 % PLGA 50:50 polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

9, 12, 15 = sonicate output

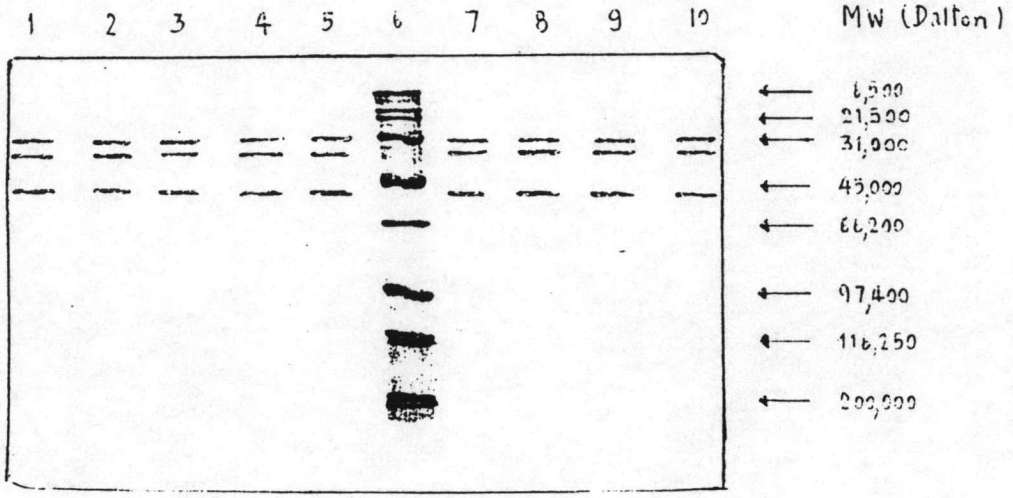


Figure 67 Structural integrity of JE antigen encapsulated in PLGA microparticles kept at 40°C for 1 month ; pure JE antigen : lane 5 , JE antigen from DL-PLA : lane 1,2 , PLGA 85:15 : lane 3,4 , PLGA 75:25 : lane 7,8 , PLGA 50:50 : lane 9,10 , and protein standard marker : lane 6

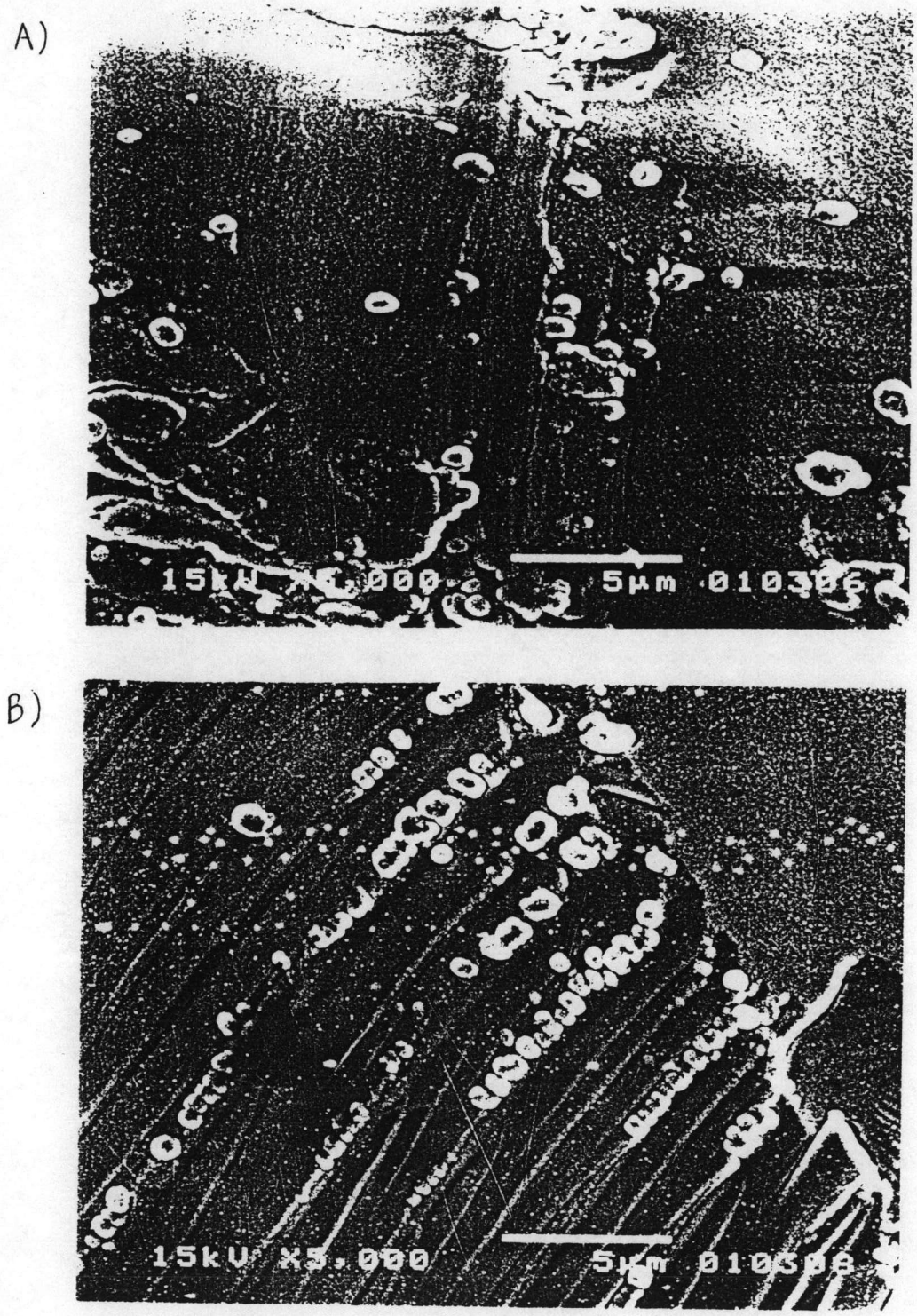


Figure 68 SEM micrographs of chitosan microparticles kept at 40°C for 1 month : A) LMW CS microparticles , B) MMW CS microparticles , C) HMW CS microparticles

C)

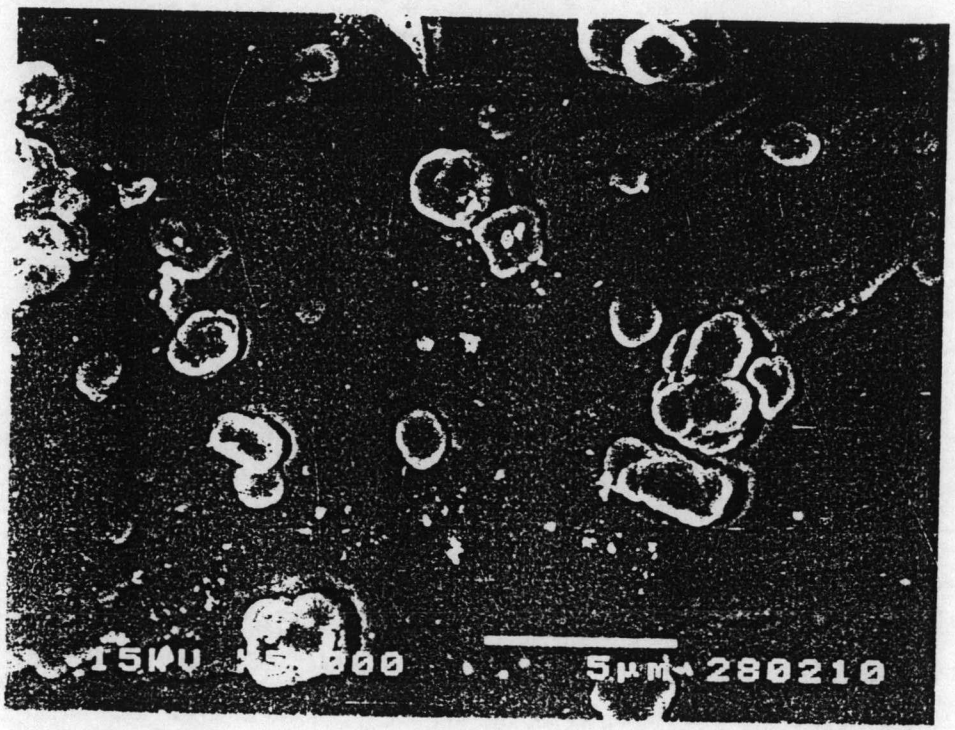


Figure 68 SEM micrographs of chitosan microparticles kept at 40°C for 1 month : A) LMW CS microparticles , B) MMW CS microparticles , C) HMW CS microparticles

Table 35 Particle size of antigen-loaded chitosan microparticles kept at 40°C for 1 month

Polymer	Concentration (%)	Ratio aqueous : oil phase	Antigen added (ug)	Particle size (um)			Uniformity
				D(v,0.1)	D(v,0.5)	D(v,0.9)	
LMW CS	1	1 : 5	750	4.81	9.63	16.70	3.840E-01
		2 : 5	750	5.47	11.19	21.66	4.425E-01
	2	1 : 5	750	2.02	12.42	72.07	2.159E+00
	3	1 : 5	750	3.48	15.52	45.67	8.907E-01
MMW CS	1	1 : 5	750	3.51	11.79	25.49	7.262E-01
	2	1 : 5	750	3.39	14.34	38.41	1.058E+00
HMW CS	1	1 : 5	750	2.91	14.08	37.94	7.731E-01

Table 36 Antigen content from chitosan microparticles kept at 40°C for 1 month

Polymer	Concentration (%)	Ratio aqueous:oil phase	Antigen added (ug)	Antigen content (ug/100 mg sample)	(SD)
LMW CS	1	1 : 5	250	99.21	(5.09)
			500	204.95	(12.77)
			750	306.70	(19.67)
		2 : 5	250	47.62	(2.24)
			500	93.83	(4.11)
			750	133.47	(7.23)
	2	1 : 5	250	48.81	(1.40)
			500	98.25	(4.25)
			750	132.44	(8.12)
	3	1 : 5	250	31.03	(1.13)
			500	59.36	(3.22)
			750	83.35	(3.95)
MMW CS	1	1 : 5	250	95.67	(5.70)
			500	189.10	(9.65)
			750	273.70	(15.16)
		2 : 5	250	43.12	(2.14)
			500	83.14	(3.78)
			750	122.57	(7.50)
	2	1 : 5	250	43.19	(1.25)
			500	87.09	(3.50)
			750	128.09	(7.21)
		2 : 5	250	28.96	(1.43)
			500	55.94	(2.19)
			750	85.60	(4.80)
HME CS	1	1 : 5	250	66.56	(2.35)
			500	147.63	(5.20)
			750	223.84	(10.80)

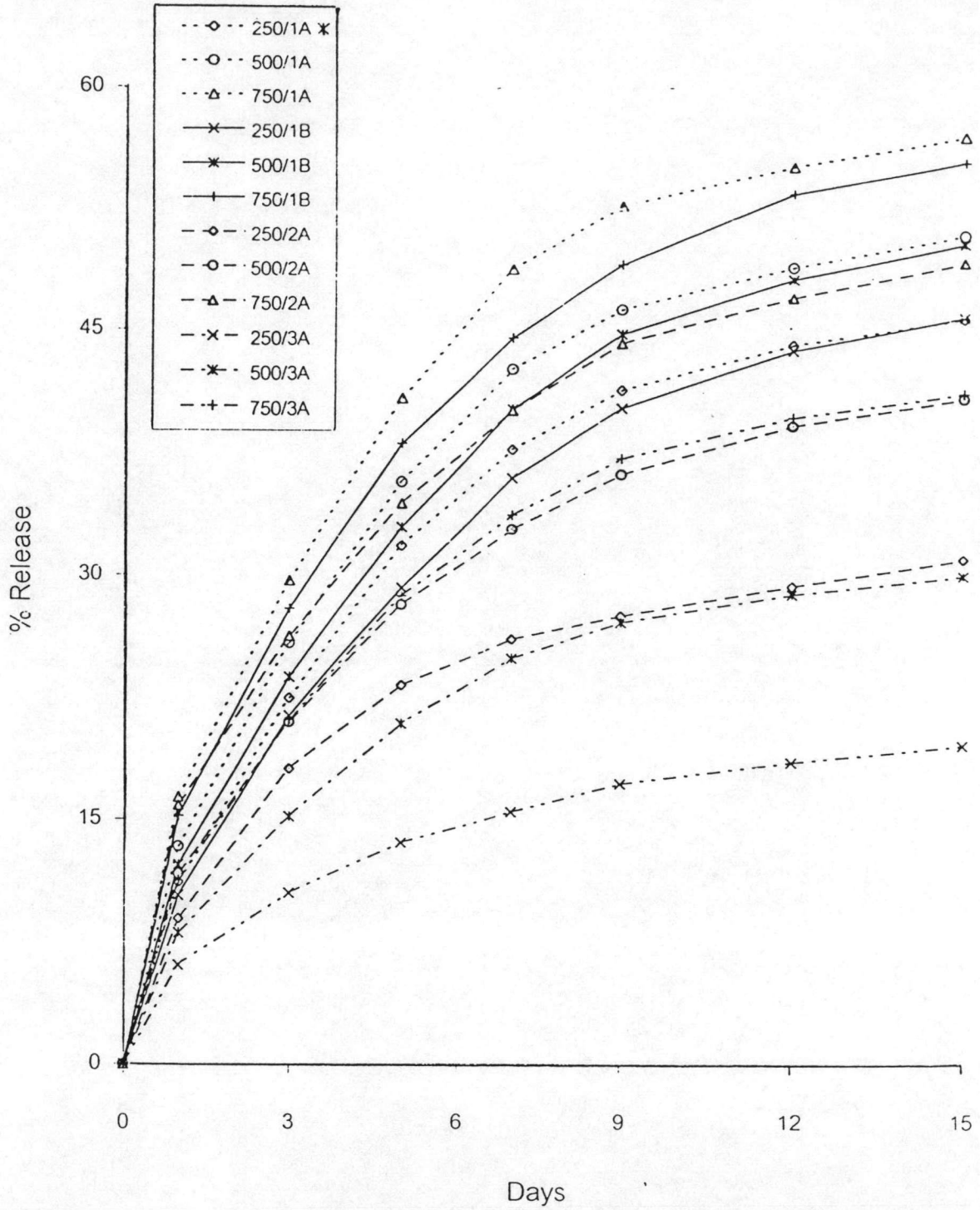


Figure 69 In vitro antigen release profiles of microparticles prepared with LMW chitosan polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

1, 2, 3 = chitosan concentration (%)

A, B = aqueous : oil phase ratio ; A = 1:5, B = 2:5

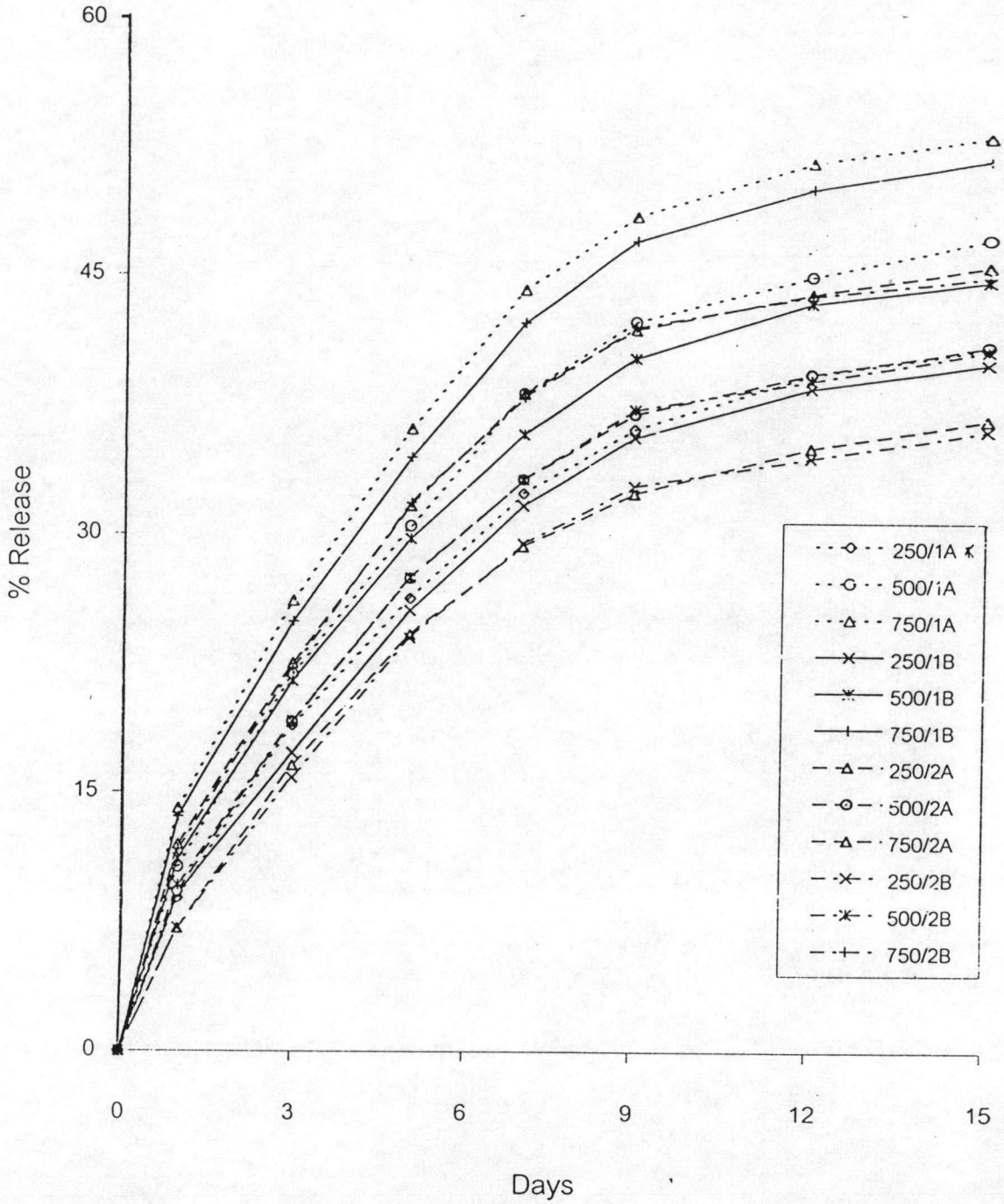


Figure 70 In vitro antigen release profiles of microparticles prepared with MMW chitosan polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

1, 2 = chitosan concentration (%)

A, B = aqueous : oil phase ratio ; A = 1:5, B = 2:5

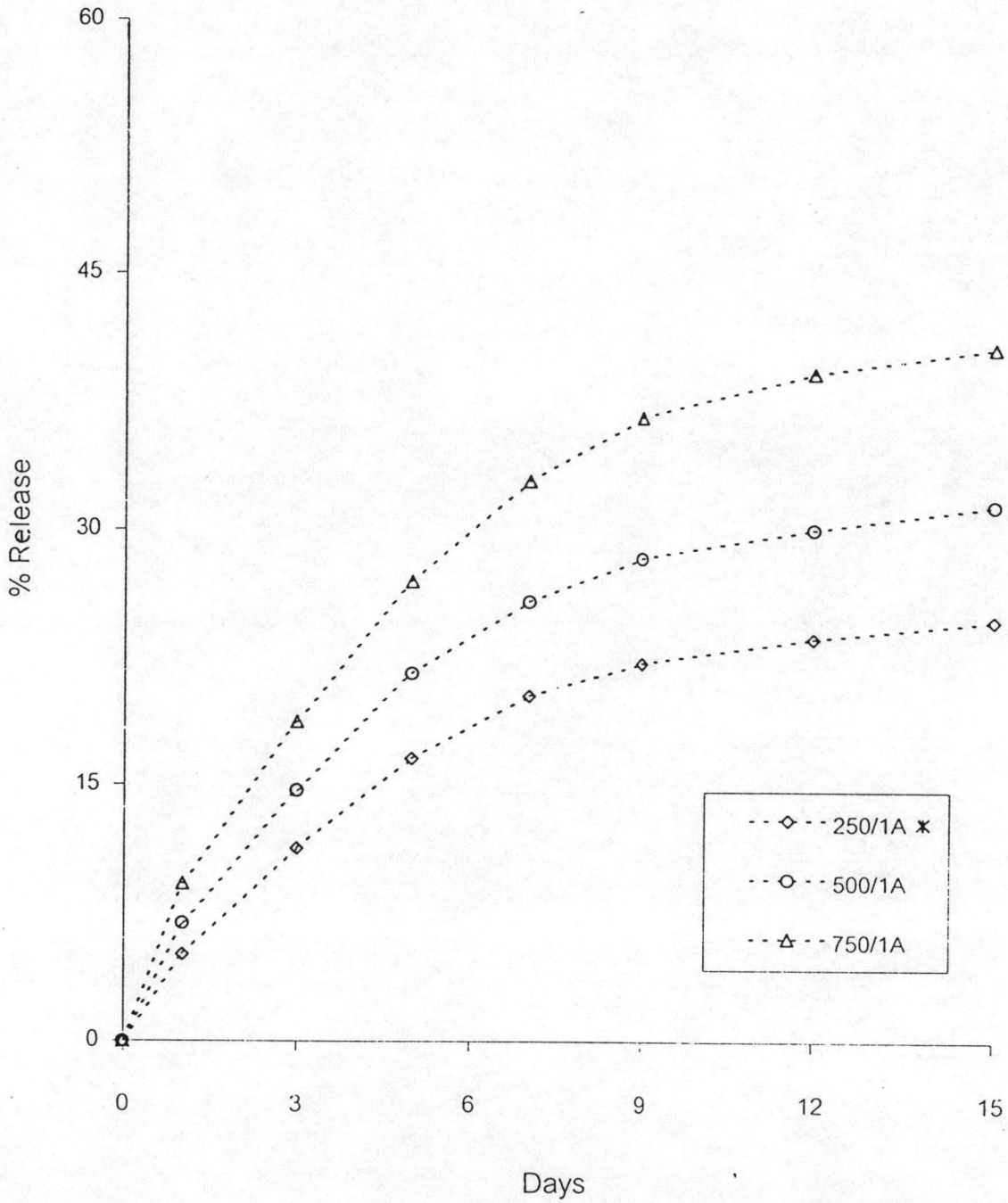


Figure 71 In vitro antigen release profiles of microparticles prepared with HMW chitosan polymer and kept at 40°C for 1 month in PBS pH 7.4

* 250, 500, 750 = amount of antigen added in formulations (ug)

1 = chitosan concentration (%)

A = aqueous : oil phase ratio ; A = 1:5

antigen was reduced after stability test. However, the reduction of release was less than 10%. This data suggested that encapsulated antigen was considerably maintained in freeze- dried chitosan microparticles after storage of 40°C for 1 month. Moreover, SDS-PAGE showed no additional band of protein as expressed in figure 72. This concluded that aggregation and smaller molecular weight fragments of proteins were not generated. Hence, the structural integrity of JE antigen still remained at high temperature.

3. Immunization study

The antibody levels induced in rabbits after subcutaneous immunization are displayed in figure 73. Each group of rabbits immunized with a single dose of 50 μg of antigen-loaded microparticles developed HI antibody titre levels higher than a group of rabbits which received a single dose of 50 μg of fluid antigen.

Immunization of a single dose of 50 μg of fluid antigen approached a maximum antibody titre level at 2 weeks and instantaneously decreased within 4 weeks. A single dose of 50 μg of antigen encapsulated in PLGA 50:50 microparticles evoked antibody titre which reached a maximum level at 10 weeks after administration. The antibody titre profile of antigen-loaded microparticles produced from 1.5% PLGA 50:50 compared to those obtained from 5% PLGA 50:50 and pure JE antigen showed that the level of antibody titre following delivery of antigen-loaded on 5% PLGA 50:50 microparticles extremely increased in antibody titre in early time period until approached a maxium level at week 10 and remained relatively constant and gradually declined until week 16 when the study was terminated. Whilst, administration of antigen-loaded on 1.5% PLGA 50:50 microparticles induced greater antibody level in early stage then reached maximum level at similar time, thereafter, a progressive decline was noted until week 16.

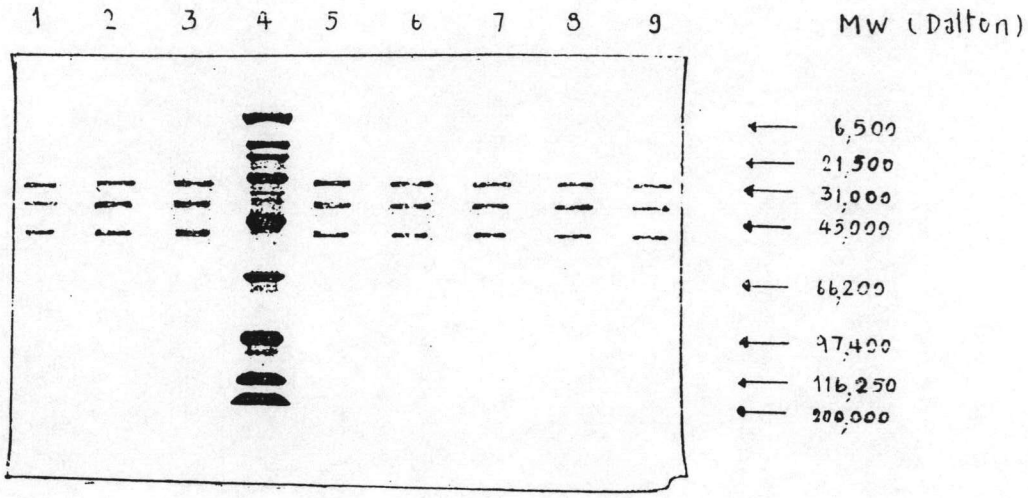


Figure 72 Structural integrity of JE antigen encapsulated in chitosan micro-particles kept at 40°C for 1 month ; pure JE antigen : lane 3,7 , JE antigen from LMW CS : lane 1,2 , MMW CS : lane 5,6 , HMW CS : lane 8,9 , and protein standard marker : lane 4

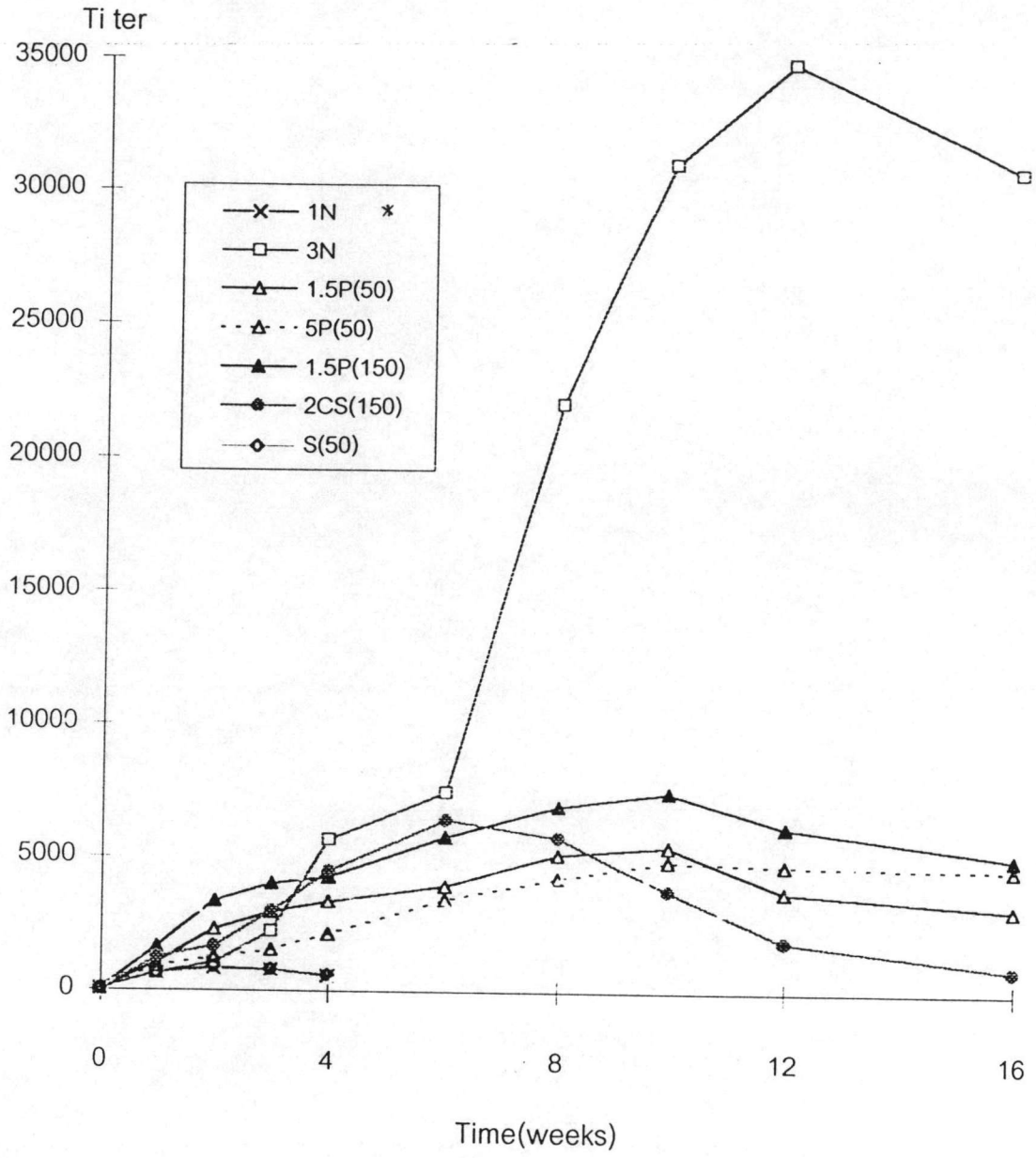


Figure 73 Antibody titre profiles of 1 ml of pure JE antigen , JE encapsulated in PLGA 50 : 50 and chitosan microparticles

* 1N : single administration of 50 μg / ml of pure JE antigen , 3N : three administrations of each 50 μg / ml of pure JE antigen , 1.5P(50) : single administration of JE encapsulated in 1.5 % PLGA 50:50 microparticles with a dose equivalent to 50 μg / ml of JE antigen , 5P(50) : single administration of JE encapsulated in 5 % PLGA 50:50 microparticles with a dose equivalent to 50 μg / ml of JE antigen , 1.5P(150) : single administration of JE encapsulated in 1.5 % PLGA 50:50 microparticles with a dose equivalent to 150 μg / ml of JE antigen , $2CS_{243}(150)$: single administration of JE encapsulated in 2% MMW chitosan microparticles with a dose equivalent to150 μg / ml of JE antigen , S : single administration of freeze-dried of JE antigen in supernatant with a dose equivalent to 50 μg / ml of JE antigen

The administration of a single dose of 150 μg of antigen-loaded on 1.5 % PLGA 50:50 microparticles was studied compared to the same preparation of a single dose of 50 μg. Both microparticle preparations showed very similar profile. However, antibody titre level from 150 μg dose elicited higher response than from 50 μg dose.

Antigen-loaded on chitosan microparticles could stimulate antibody titre level. The maximum titre peak of a single dose of 150 μg of antigen-loaded on chitosan microparticles was observed at six weeks after administration. The antibody titre profile had progressively increased, reached a maximum peak and progressively decreased along the study period. Although, antigen-loaded on chitosan microparticles could evoke antibody response but they gave lower titre level and less prolong effect comparable to that obtained from PLGA 50:50 microparticles with the same dose.